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Dianne M. Rees, Ph.D
Edwards & Angell, LLP
PO Box 9169
Boston, MA 02209

| EXAMINER | |
|---|---|
| CROW, ROBERT THOMAS | |
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Please find below and/or attached an Office communication concerning this application or proceeding.

| Office Action Summary | Application No. | Applicant(s) | |
|---|---|---|---|
| | 10/601,140 | KAUPPINEN ET AL. | |
| | Examiner | Art Unit | |
| | Robert T. Crow | 1634 | |

*-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --*

**Period for Reply**

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

**Status**

1)☒ Responsive to communication(s) filed on *21 April 2006*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

**Disposition of Claims**

4)☒ Claim(s) *1-140* is/are pending in the application.

4a) Of the above claim(s) *23,24,81-89,94-109 and 120-126* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-22,25-80,90-93,110-119 and 127-140* is/are rejected.

7)☒ Claim(s) *9,17,55,92 and 138* is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

**Application Papers**

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on *20 June 2003* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

**Priority under 35 U.S.C. § 119**

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

**Attachment(s)**

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

## DETAILED ACTION

### *Election/Restrictions*

Applicant's election with traverse of Group I and species SEQ ID NO: 3 in the reply filed on 21 April 2006 is acknowledged. The traversal is on the ground(s) that the search would not be burdensome. These arguments are not found persuasive because, as stated on page 5 of the Requirement for Restriction/Election, the inventions have acquired a separate status in the art because of their recognized divergent subject matter as exemplified by their different classification. Furthermore, a search for the inventions of all of the groups would not be co-extensive because a search indicating the ***process is*** novel or nonobvious would not extend to a holding that the ***product itself is*** novel or nonobvious; similarly, a search indicating that ***the product is*** known or would have been obvious would not extend to a holding that ***the process is*** known or would have been obvious.

The requirement is still deemed proper and is therefore made FINAL.

Claims 81-89, 94-109, and 120-126 are therefore withdrawn. Claims 23-24 are withdrawn, as claim 23 is drawn to a non-elected sequence. Claims 25 and 26 are interpreted as being drawn the elected sequence SEQ ID NO. 3 (i.e., compound 3).

Claims 1-22, 25-80, 90-93, 110-119, and 127-140 are currently under prosecution.

*Claim Objections*

1. The numbering of claims is not in accordance with 37 CFR 1.126 which requires the original numbering of the claims to be preserved throughout the prosecution. When claims are canceled, the remaining claims must not be renumbered. When new claims are presented, they must be numbered consecutively beginning with the number next following the highest numbered claims previously presented (whether entered or not).

Misnumbered claims 87-135 been renumbered 92-140 and have been corrected for dependency. In addition, renumbered claim 93 is interpreted as being dependent on renumbered claim 92.

2. Claims 9, 17, 55, 92, and 138 are objected to because of the following informalities:

A. The amendment to claim 9, i.e., "[capture probe/," begins with a bracket but does not end with a bracket. Appropriate correction is required.

B. The amendment to claim 17, i.e., "[ probe/," begins with a bracket but does not end with a bracket. Appropriate correction is required.

C. Claim 55 recites "the said LNA" in line 3 of the claim. This redundancy appears to be a typographical error. Appropriate correction is required.

D. Claim 92 recites "a target nucleic acid molecule the nucleotide sequence which" in lines 1-2 of the claim. This appears to be a typographical error. Appropriate correction is required.

3. Claim 138 is objected to under 37 CFR 1.75 as being a substantial duplicate of claim 137. When two claims in an application are duplicates or else are so close in content that they both cover the same thing, despite a slight difference in wording, it is proper after allowing one claim to object to the other as being a substantial duplicate of the allowed claim. See MPEP § 706.03(k).

*Claim Rejections - 35 USC § 112*

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 9-15, 22, 27-29, 33-34, 37-38, 42-43, 45-47, 58, 59, 113, and 117 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

1. Claim 9 is indefinite in the recitation "a homopolymeric nucleotide" in line 2 of the claim. It is unclear how a nucleotide, which comprises a single base, is polymeric.

2. Claims 9-15, 27-29, 33-34, and 45-47 are indefinite in the recitation "at least about " in lines 2-3 of claim 9 and in line 2 of each of claims 10-15, 27-29, 33-34, and 45-47. The phrase "at least" typically indicates a minimum point; however, the phrase "at least" is controverted by the term "about," which implies that values above and below the indicated amount are permitted. Therefore, the juxtaposition of these two terms makes it unclear what minimum purity is encompassed by the claim. In Amgen, Inc. v. Chugai Pharmaceutical co., 927 F.2d 1200 (CAFC 1991), the CAFC stated, "[t]he district court held claims 4 and 6 of the patent invalid because their specific activity of "at least about 160,000" was indefinite." After review, the CAFC states "[w]e therefore affirm the district court's determination on this issue." Thus, the CAFC found the phrase "at least about" indefinite where the metes and bounds of the term were not defined in the Specification.

3. Claim 22 is indefinite in the recitation "the LNA is 5'-biotin-TttTttTttTttTttTttTt." Claim 22 is dependent upon 18, wherein the LNA is complementary to a sequence consisting substantially of a poly(T) sequence (i.e., the LNA has a poly (A) sequence). It is unclear how 5'-biotin-TttTttTttTttTttTttTt is complementary to a sequence consisting substantially of a poly(T) sequence.

4. Claim 37 is indefinite in the recitation "the association constant (Ka) of the LNA oligonucleotide is higher" in lines 1-2 of the claim. It is unclear if the association

constant is for the LNA with itself or a complementary sequence. Claim 37 is also indefinite in the recitation (Ka) because it is unclear if (Ka) within the parenthesis is a limitation of the claim.

5. Claim 38 is indefinite in the following:

A. The recitation "the association constant (Ka) of the LNA oligonucleotide is higher" in lines 1-2 of the claim. It is unclear if the association constant is for the LNA with itself or a complementary sequence. There is also insufficient antecedent basis for this limitation of the claim. It is suggested that "the association constant" be changed to "a association constant."

B. The recitation "(Ka)" because it is unclear if (Ka) within the parenthesis is a limitation of the claim.

C. The recitation "the dissociation constant (Kd) of the complementary strand of the target sequence in a double stranded molecule" in lines 2-3 of the claim. It is unclear what the if the Kd refers to dissociation of the double stranded target or if Kd refers to dissociation of the target from a different double stranded molecule (e.g., a hybrid between the target and an antibody). There is also insufficient antecedent basis for this limitation of the claim. It is suggested that "the dissociation constant" be changed to "a dissociation constant."

D. The recitation "(Kd)" because it is unclear if (Kd) within the parenthesis is a limitation of the claim.

6. Claim 42 is indefinite in the recitation "the fluorescent signal" in lines 4-5 of the claim. There is insufficient antecedent basis for this limitation in the claim. It is suggested that the word "the" be changed to "a." Claim 42 is also indefinite in the recitation "the fluorescent signal from the nucleotide" at the end of the claim. There is insufficient antecedent basis for "the nucleotide" in the claim. It is also unclear how the nucleotide (i.e., a single base) creates a fluorescent signal.

7. Claims 43 and 113 are indefinite in the recitation "the Tm" in line one of each of the claims. There is insufficient antecedent basis for this limitation in the claim. It is suggested the word "the" e changed to "a."

8. Claim 58 is indefinite in the following:

A. The recitation "LNA containing a complementary overhang to a free arm in a dendrimer or a branched oligonucleotide" in lines 2-3 of the claim. It is unclear if "or" indicates that the branched oligonucleotide is a free arm contained in the complementary overhang or if the branched oligonucleotide is merely contained in the LNA.

B. The recitation "or biotin molecules" in lines 4-5 of the claim. It is unclear if the biotin molecules are an alternative to the complementary overhang or if the biotin molecules are an alternative to e.g., fluorescein isothiocyante.

C. The recitation "or fluorochrome molecules" in line 5 of the claim. It is unclear if the fluorochrome molecules are an alternative to the complementary overhang or if the fluorochrome molecules are an alternative to e.g., fluorescein isothiocyante.

D. The remaining recitations of the last 4 lines of the claim. The placement of the word "or" in lines 6 and 7 of the claim renders the claim unclear as to what the exact alternative embodiments are. It is suggested that the claim be amended to list the alternative embodiments similar to the format of claim 56.

9. Claim 59 is indefinite in the recitation "the sample" in the last line of the claim. There is insufficient antecedent basis for this limitation in the claim. It is suggested the claim be amended to reflect proper antecedent basis.

10. Claims 110-119 are indefinite in claim 110, which recites the limitation "the components" in line 7 of claim 110. There is insufficient antecedent basis for this limitation in the claim. It is suggested the claim be amended to reflect proper antecedent basis. Claims 110-119 are also indefinite in claim 110, which recites the limitation "the capturing probe" in line 10 of claim 110. There is insufficient antecedent basis for "the capturing probe" in "a capture probe." It is suggested "capturing" be changed to "capture."

11. Claim 117 is indefinite in the recitation "the retrovirus" in line 1 of the claim. There is insufficient antecedent basis for "the retrovirus" in "retroviruses" as found in claim 116. It is suggested the claim be amended to reflect proper antecedent basis.

*Claim Rejections - 35 USC § 102*

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless -
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 1-3, 5-11, 16, 18-21, 27, 29, 30, 33-43, 53-54, 74-76, and 91-92 are rejected under 35 U.S.C. 102(b) as being anticipated by Wengel et al (PCT International Publication No. WO 99/14226, published 25 March 1999).

Regarding claim 1, Wengel et al teach a method for detecting an/or isolating a target nucleic acid molecule having a homopolymeric sequence comprising: treating a sample containing nucleic acid molecules with an LNA oligonucleotide to thereby detect and/or isolate a nucleic acid molecule having said homopolymeric sequence (e.g., poly dT primers containing LNA T residues are used to prime cDNA synthesis; page 181, Example 160).

Regarding claim 2, Wengel et al teach a method for detecting an/or isolating a target nucleic acid molecule having a repetitive element comprising: treating a sample

containing nucleic acid molecules with an LNA oligonucleotide to thereby detect and/or isolate a nucleic acid molecule having the repetitive element (e.g., poly dT primers containing LNA T residues are used to prime cDNA synthesis; page 181, Example 160).

Regarding claim 3, Wengel et al teach a method for detecting an/or isolating a target nucleic acid molecule having a conserved polynucleotide sequence comprising: treating a sample containing nucleic acid molecules with an LNA oligonucleotide to thereby detect and/or isolate a nucleic acid molecule having the conserved polynucleotide sequence (e.g., poly dT primers containing LNA T residues are used to prime cDNA synthesis; page 181, Example 160).

Regarding claim 5, Wengel et al teach the method of claim 1 wherein the LNA oligonucleotide is covalently attached to a solid support (e.g., LNA oligonucleotides are immobilized on a solid support; page 15, lines 7-9).

Regarding claim 6, Wengel et al teach the method of claim 1 wherein the LNA oligonucleotide is synthesized with an anthraquinone moiety and a linker at the at the 5'-end of the 3'-end of said oligonucleotide (e.g., the oligonucleotides carry an anthraquinone and a linker; page 62, lines 15-27).

Regarding claim 7, Wengel et al teach the method of claim 6 wherein the said linker is a higher hexaethylene glycol polymer (e.g., polyethylene glycol; page 21, lines 10-15).

Regarding claim 8, Wengel et al teach the method of claim 5 wherein said solid support is a polymer support is a polystyrene bead (page 62, lines 15-18).

Regarding claim 9, Wengel et al teach the method of claim 1, wherein the LNA oligonucleotide is complementary to a homopolymeric nucleotide comprising at least about one nucleobase that is different than the bases comprising the homopolymeric nucleic acid sequence (e.g., Probe RTZ5, 5'-**TTTTTTTTT**T [page 181, Example 160], wherein the LNA residues are in bold, and wherein the probe is complementary to a mRNA having a poly(A) tail [i.e., the poly (A) tail is the homopolymeric nucleotide] and the remainder of the mRNA has at least one nucleobase that is different from A; i.e., the message has the start codon AUG, which has bases other than A, which is the homopolymeric nucleic acid sequence).

Regarding claims 10-11, Wengel et al teach the method of claim 1 wherein the LNA oligonucleotide comprises at least about 10 repeating consecutive nucleotides (e.g., a poly dT LNA primer that is 15 bases long; page 181, lines 15-20).

Regarding claim 16, Wengel et al teach the method of claim 1 wherein the LNA oligonucleotide is complementary to a nucleotide sequence consisting substantially of a poly(A) nucleotide sequence (e.g., the LNA is a poly dT LNA; page 181, lines 15-20).
Regarding claim 6, Wengel et al teach the method of claim 1 wherein the LNA oligonucleotide is synthesized with an anthraquinone moiety and a linker at the at the 5'-end of the 3'-end of said oligonucleotide (e.g., the oligonucleotides carry an anthraquinone and a linker; page 62, lines 15-27).

Regarding claim 18, Wengel et al teach the method of claim 1 wherein the LNA oligonucleotide is complementary to a nucleotide sequence consisting substantially of a poly(T) nucleotide sequence (e.g., page 175, Example 154, wherein the LNA oligo 5'-d(**TGTGTGAAATTGTTA**) is complementary to dTTT, and wherein those bases in bold represent LNA nucleotides).

Regarding claim 19, Wengel et al teach the method of claim 1 wherein the LNA oligonucleotide is complementary to a nucleotide sequence consisting substantially of a poly(G) nucleotide sequence (e.g., page 163, Example 146, wherein the LNA oligo biotin-5'-d(**TTCCACAGCACA**A) is complementary to dGG).

Regarding claim 20, Wengel et al teach the method of claim 1 wherein the LNA oligonucleotide is complementary to a nucleotide sequence consisting substantially of a poly(U) nucleotide sequence (e.g., page 175, Example 154, wherein the LNA oligo 5'-d(**TGTGTGAAATTGTTA**) is complementary to rUUU).

Regarding claim 21, Wengel et al teach the method of claim 1 wherein the LNA oligonucleotide is complementary to a nucleotide sequence consisting substantially of a poly(C) nucleotide sequence (e.g., page 152, Example 137, wherein the LNA oligo 5'-d(GGTGG**TTTG**TTTG) is complementary to dCC).

Regarding claim 27, Wengel et al teach the method of claim 2 wherein the LAN oligonucleotide is complementary to a repetitive nucleotide sequence comprising at least one base that is different that the bases comprising the repetitive sequence (e.g.,

Seq. No 131, 5'-GTGTGGAT, which is complementary to 3'CACACACA except for the 2 base run of GA at the 3'end; page 169).

Regarding claim 29, Wengel et al teach the method of claim 1, wherein the LNA oligonucleotide is comprises at least one nucleotide having a nucleobase that is different from the nucleobases of the remaining oligonucleotide sequence (e.g., Probe No ATZ-1, 5'-Cy3-**TTCCACAC**; wherein the A residues are different from the T and C residues of the rest of the sequence; page 171, line 23).

Regarding claim 30, Wengel et al teach the method of claim 1, wherein the -1 residue of the LNA oligonucleotide molecule 3' and/or 5' end is an LNA residue (e.g., Probe No ATZ-1, 5'-Cy3-**TTCCACAC**; wherein the second T residue is an LNA; page 171, line 23).

Regarding claim 33, Wengel et al teach the method of claim 1, wherein the LNA oligonucleotide comprises at least about 20 to 50 percent LNA residues based on total residues of the LNA oligonucleotide (e.g., Probe No ATZ-1, 5'-Cy3-**TTCCACAC**; wherein 4 of the 8 residues are LNA; page 171, line 23).

Regarding claim 34, Wengel et al teach the method of claim 1, wherein the LNA oligonucleotide comprises at least about 2 or more consecutive LNA molecules (e.g., Probe No ATZ-1, 5'-Cy3-**TTCCACAC**; page 171, line 23).

Regarding claim 35, Wengel et al teach the method of claim 1, wherein the LNA oligonucleotide comprises modified and non-modified nucleotide molecules (e.g., Probe

No ATZ-1, 5'-Cy3-**TTCCACAC**; page 171, line 23, which has both LNA and non-LNA residues).

Regarding claim 36, Wengel et al teach the method of claim 1, wherein the LNA probe comprises a compound of the formula 5'-$Y^q$-$(X^p$-$Y^n)_m$-$X^p$Z-3' (e.g., Probe RTZ5, 5'-**TTTTTTTTTT**, where q=0, n=0, the first p = 6, the second p = 3, and Z is a dT residue at the 3' end; page 181, Example 160).

Regarding claim 37, Wengel et al teach the method of claim 1 wherein the association constant of the LNA oligonucleotide is higher that the association constant of the complementary strands of a double stranded molecule (e.g., LNA has a positive effect of the thermal stability of duplexes towards DNA and RNA; page 151, Example 135).

Regarding claim 38, Wengel et al teach the method of claim 1 wherein the association constant of the LNA oligonucleotide is higher that the dissociation constant of the complementary strand in a double stranded molecule (e.g., the LNA performs strand displacement on dsDNA; page 59, lines 23-29).

Regarding claim 39, Wengel et al teach the method of claim 1, wherein the LNA oligonucleotide is complementary to the sequence it is designed to detect and/or isolate (e.g., LNA-modified oligonucleotides function efficiently in the sequence specific capture of RNA molecules; page 182, lines 23-24).

Regarding claim 40, Wengel et al teach the method of claim 1, wherein the LNA oligonucleotide has at least one base pair difference to a complementary sequence it is

designed to detect and/or isolate (e.g., the LNA molecules detect end mismatches; page 170, example 152).

Regarding claim 41, Wengel et al teach the method of claim 40, wherein the LNA oligonucleotide can detect at least about one base pair difference between a complementary poly-repetitive sequence and the LNA/DNA oligonucleotide (e.g., the LNA molecules detect end mismatches [page 170, example 152], and the probes include poly repetitive sequences (e.g., Probe RTZ5, 5'-**TTTTTTTTTT**; page 181, Example 160).

Regarding claim 42, Wengel et al teach the method of claim 1, wherein the LNA oligonucleotide comprises a fluorophore moiety and a quencher moiety, positioned in such a way that a hybridized state of the oligonucleotide can be distinguished from an unbound state of the oligonucleotide by an increase in the fluorescent signal from the nucleotide (e.g., the LNA is a molecular beacon; page 64, lines 1-25).

Regarding claim 43, Wengel et al teach the method of claim 1, wherein the Tm of the LNA oligonucleotide is between about 50°C to about 70°C when the LNA oligonucleotide hybridizes to its complementary sequence (e.g., Tm No. 10, Table 1, page 184).

Regarding claim 53, Wengel et al teach the method of claim 1 wherein the LNA oligonucleotide is adapted for use an a molecular beacon (page 64, lines 1-25).

Regarding claim 54, Wengel et al teach the method of claim 1 wherein the LNA oligonucleotide hybridizes to complementary sequences of eukaryotic RNA (e.g., the

LNA are used to activate genes of therapeutic interest [page 60, lines 21-24], wherein the therapeutic applications are in humans; page 18, lines 5-6).

Regarding claim 74, Wengel et al teach the method of claim 1, wherein the LNA oligonucleotide comprises a fluorescent reporter molecule at one end of the molecule and a quencher molecule at a second end and wherein the reporter molecule is quenched by the quencher molecule with the LNA oligonucleotide is not hybridized to the nucleic acid molecules (e.g., the LNA is a molecular beacon, wherein the reporter [i.e., fluorophore] and quencher are at the ends of the molecule; page 64, lines 1-25).

Regarding claim 75, Wengel et al teach the method of claim 74, wherein hybridization of the LNA oligonucleotide is detected by detecting the increased fluorescence of the reporter molecules (page 64, lines 1-25).

Regarding claim 76, Wengel et al teach the method of claim 74, wherein the LNA oligonucleotide comprises, in addition to a sequence sufficiently complementary to said nucleic acid molecule to specifically hybridize to said nucleic acid molecule, a first and second complementary sequence which specifically hybridize to each other when the oligonucleotide is not hybridized to the nucleic acid molecule, bringing said quencher molecule in sufficient proximity to said reporter molecule to quench fluorescence of the reporter molecule (e.g., the molecular beacon forms a hairpin in the absence of the target; page 64, lines 1-10).

Regarding claim 91, Wengel et al teach the method of claim 54, wherein the LNA oligonucleotide hybridized to complementary sequences of mRNA (e.g., poly dT

probes [i.e., primers] containing LNA T residues are used to prime Arabidopsis mRNA; page 181, Example 160).

Regarding claim 92, Wengel et al teach a method for amplifying a target nucleic acid molecule the nucleotide sequence which is complementary to a LNA oligonucleotide capture probe, the method comprising: providing a sample containing nucleic acid molecules having repetitive base sequences (e.g., a sample with an sequence that binds to a first primer 5'-GGTGG**TTTG**TTTG-3', which contains the complement to the repetitive sequence GGTGGT; page 165, lines 10-15); and contacting the nucleic acid molecules from the sample with at least one LNA oligonucleotide capture probe to capture target nucleic acids (e.g., the LNA probe hybridizes to the targets; page 195, Example 148); and subjecting the captured nucleic acids to polymerase chain reaction, using primers to amplify the captured nucleic acid (e.g., the target is amplified in a PCR amplification using an additional primer in addition to the first primer [page 195, Example 148], and wherein captured nucleic acids are amplified directly on a surface; page 62, lines 4-10).

***Claim Rejections - 35 USC § 103***

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

1. This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

2. Claims 1, 12-15, and 17 are rejected under 35 U.S.C. 103(a) as being unpatentable over Wengel et al (PCT International Publication No. WO 99/14226, published 25 March 1999) in view of Monforte et al (U.S. Patent No. 6,051,378, issued 18 April 2000).

Regarding claims 12-15, Wengel et al teach the method of claim 1 for detecting an/or isolating a target nucleic acid molecule having a homopolymeric sequence comprising: treating a sample containing nucleic acid molecules with an LNA oligonucleotide to thereby detect and/or isolate a nucleic acid molecule having said homopolymeric sequence (e.g., poly dT primers containing LNA T residues are used to prime cDNA synthesis; page 181, Example 160). While Wengel et al teach repeating nucleotides (e.g., poly dT primers containing LNA; page 181), Wengel et al do not teach oligonucleotides comprising at least about fifty nucleotides.

However, Monforte et al teach hybridization using capture probes that are 50 nucleotides in length with the added advantage that they provide stability to the hybridization of the capture probe (column 19, lines 40-42).

It would therefore have been obvious to a person of ordinary skill in the art at the time the invention was claimed to have modified the method as taught by Wengel et al with the probe lengths as taught by Monforte with a reasonable expectation of success. The ordinary artisan would have been motivated to make the modification because the modification would have provided stability to the hybridization of the capture probe as explicitly taught by Monforte et al (column 19, lines 40-42).

Regarding claim 17, the method of claim 15 is discussed above. Wengel et al also teach the LNA oligonucleotide is synthesized with an anthraquinone moiety and a linker at the 5'-end of said oligonucleotide (e.g., the oligonucleotides carry an anthraquinone and a linker; page 62, lines 15-27), wherein said linker is a higher hexaethylene glycol polymer (e.g., polyethylene glycol; page 21, lines 10-15). While Wengel et al teach LNA oligonucleotides covalently coupled to a solid polymer support (e.g., LNA oligonucleotides are immobilized on a solid support; page 15, lines 7-9) via excitation of the anthraquinone moiety using UV light (page 62, lines 23-26 and page 166, Example 149), Wengel et al do not specifically teach immobilization of the poly dT primers.

However, Wengel et al do teach that immobilization of nucleic acids is preferred because it allows hybridization and capture to occur simultaneously (page 62, lines 4-10).

It would therefore have been obvious to a person of ordinary skill in the art at the time the invention was claimed to have modified the method of claim 1 as taught by Wengel et al by immobilizing the LNA molecules as also taught by Wengel with a reasonable expectation of success. The ordinary artisan would have been motivated to make the modification because the modification would have resulted in simultaneous hybridization and capture as explicitly taught by Wengel et al (page 62, lines 4-10).

3. Claims 1, 2, 4, 22, 25-26, 28, 44-52, and 127-135 are rejected under 35 U.S.C. 103(a) as being unpatentable over by Wengel et al (PCT International Publication No. WO 99/14226, published 25 March 1999) in view of Skouv (U.S. Patent No. 6,303,315 B1, issued 16 October 2001).

Regarding claim 4, the method of claim 1 is discussed above. Wengel et al do not teach chaotropic agents.

However, Skouv et al teach the preparation of nucleic acids from biological samples using LNA and lysing in the presence of chaotropic agents (Abstract) with the added advantage that chaotropic agents facilitate lysis of the cells (column 1, lines 38-42).

It would therefore have been obvious to a person of ordinary skill in the art at the time the invention was claimed to have modified to method as taught by Wengel et al with the chaotropic agent as taught by Skouv with a reasonable expectation of success. The ordinary artisan would have been motivated to make such a modification because said modification would have resulted in facilitation of the lysis of the cells as explicitly taught by Skouv (column 1, lines 38-42).

Regarding claim 22, the method of claim 1 is discussed above. Wengel et al also teach the LNA oligonucleotide is complementary to a nucleotide sequence consisting substantially of a poly(T) nucleotide sequence (e.g., page 175, Example 154, wherein the LNA oligo 5'-d(**TGTGTGAAATTGTTA**) is complementary to dTTT, and wherein those bases in bold represent LNA nucleotides). Wengel et al also teach oligo dT comprising (Table, page 183), as well as biotinylated LNA (page 19, lines 8-10), but do not specifically teach LNA at every third residue (i.e., compound 3 [SEQ ID NO. 3].

However, Skouv teaches an LNA oligomer having the formula (column 23)

$$G\text{-}[Nu\text{-}L]_{n(0)}\text{-}\{[LNA\text{-}L]_{m(q)}\text{-}[Nu\text{-}L]_{n(q)}\}_{q}\text{-}G^{*} \qquad V$$

wherein

q is 1–50;

each of n(0), . . . , n(q) is independently 0–10000;

each of m(1), . . . , m(q) is independently 1–10000;

with the proviso that the sum of n(0), . . . , n(q) and m(1), . . . , m(q) is 2–15000;

G designates a 5'-terminal group;

each Nu independently designates a nucleoside selected from naturally occurring nucleosides and nucleoside analogues;

each LNA independently designates a nucleoside analogue;

each L independently designates an internucleoside linkage between two groups selected from Nu and LNA, or L together with G* designates a 3'-terminal group; and

each LNA-L independently designates a nucleoside analogue of the general formula I as defined above.

which encompasses the alternating pattern of the instant claim, with the added advantage that the oligomers have good affinity and specificity in hybridization (column 24, lines 5-7).

It would therefore have been obvious to a person or ordinary skill in the art at the time the invention was claimed to have modified to method as taught by Wengel et al with the alternating LNA residues as taught by Skouv with a reasonable expectation of success. The ordinary artisan would have been motivated to make such a

modification because said modification would have resulted in good affinity and specificity in hybridization as explicitly taught by Skouv et al (column 24, lines 5-7).

Regarding claim 25, the method of claim 18 and compound 3 [SEQ ID NO. 3] are discussed above. Wengel et al also teach the LNA oligonucleotide is synthesized with an anthraquinone moiety and a linker at the 5'-end of said oligonucleotide (e.g., the oligonucleotides carry an anthraquinone and a linker; page 62, lines 15-27), wherein said linker is a higher hexaethylene glycol polymer (e.g., polyethylene glycol; page 21, lines 10-15).

Regarding claim 26, the method of claim 18 and compound 3 [SEQ ID NO. 3] are discussed above. Skouv et al also teach LNA without biotin substitutions (e.g., the group G in the formula is a 5' terminal group).

Regarding claim 28, Wengel et al teach the method of claim 2 for detecting an/or isolating a target nucleic acid molecule having a conserved nucleotide sequence comprising: treating a sample containing nucleic acid molecules with an LNA oligonucleotide to thereby detect and/or isolate a nucleic acid molecule having the repetitive element (e.g., poly dT primers containing LNA T residues are used to prime cDNA synthesis; page 181, Example 160). Wengel et al do not teach one base different from the conserved sequence.

However, Skouv teaches the detection of at least one base different in a conserved region (e.g., missense mutations in conserved regions of highly conserved

regions of TP53) with the added advantage that missense mutations are indicative of tumors (column 2, lines 5-13).

It would therefore have been obvious to a person of ordinary skill in the art at the time the invention was claimed to have modified the method of Wengel et al with the at least one base differences as taught by Skouv with a reasonable expectation of success. The ordinary artisan would have been motivated to make such a modification because said modification would have resulted in indicating the presence of tumors as explicitly taught by Skouv (column 2, lines 5-13).

Regarding claim 44, the method of claim 4 is discussed above. Skouv also teaches the chaotropic reagent is guanidinium thiocyanate (column 21, line 65-column 22, line 8).

Regarding claims 45-47, the method of claim 44 is discussed above. Skouv also teaches the concentration of guanidinium thiocyanate is at least about 4M (column 22, lines 1-8).

Regarding claim 48, the method of claim 44 is discussed above. Skouv also teaches hybridization at a temperature in the range of 20-65 °C (e.g., 37 °C; column 36, lines 40-50).

Regarding claim 49, the method of claim 48 is discussed above. The courts have stated where the claimed ranges "overlap or lie inside the ranged disclosed by the prior art" and even when the claimed ranges and prior art ranges do not overlap but are close enough that one skilled in the art would have expected them to have similar properties,

a *prima facie* case of obviousness exists (see *In re Wertheim*, 541 F.2d 257, 191 USPQ 90 (CCPA 1976); *In re Woodruff*, 919 F.2d 1575, 16 USPQ2d 1934 (Fed. Cir. 1990); *Titanium Metals Corp. of America v. Banner*, 778 F2d 775. 227 USPQ 773 (Fed. Cir. 1985) (see MPEP 2144.05.01). Therefore, the claimed ranges "at about 20 °C" is obvious over the 37 °C as taught by Skouv (column 36, lines 40-50).

Regarding claim 50, the method of claim 48 is discussed above. Skouv also teaches hybridization at a temperature of about 37 °C (column 36, lines 40-50).

Regarding claims 51 and 52, the method of claim 48 is discussed above. As stated above, when the claimed range is close enough that one skilled in the art would have expected them to have similar properties, a *prima facie* case of obviousness exists. Therefore, the claimed temperatures of about 55 °C and about 60 °C are obvious over the temperatures taught by Skouv (e.g., 37 °C [column 36, lines 40-50], and 70-100 °C; column 10, lines 53-56).

Regarding claim 127, the method of claim 1 is discussed above. While Wengel et al also teach high stringency hybridization at low salt concentration (e.g., selective binding occurs in low salt; page 183), Wengel et al do not teach chaotropic agents.

However, Skouv et al teach the isolation (i.e., preparation) of nucleic acids from biological samples using LNA and lysing in the presence of chaotropic agents (Abstract) with the added advantage that chaotropic agents facilitate lysis of the cells (column 1, lines 38-42).

It would therefore have been obvious to a person of ordinary skill in the art at the time the invention was claimed to have modified to method as taught by Wengel et al with the chaotropic agent as taught by Skouv with a reasonable expectation of success. The ordinary artisan would have been motivated to make such a modification because said modification would have resulted in facilitation of the lysis of the cells as explicitly taught by Skouv (column 1, lines 38-42).

Regarding claims 128, the method of claim 127 is discussed above. Skouv also teaches the concentration of guanidinium thiocyanate is at least about 4M (column 22, lines 1-8).

Regarding claim 129, the method of claim 127 is discussed above. Skouv also teaches a binding buffer containing NaCl (column 4, lines 53-57).

Regarding claims 130-132, the method of claim 129 is discussed above. Skouv also teaches the NaCl concentration is less than 25 mM (e.g., between about 0 and 1M; column 4, lines 53-57).

Regarding claims 133-134, the method of claim 127 is discussed above. Skouv also teaches hybridization at a temperature of at least 37 °C (column 36, lines 40-50).

Regarding claim 135, the method of claim 127 is discussed above. Skouv also teaches hybridization at a temperature of at least 50 °C (e.g., 70-100 °C; column 10, lines 53-56).

4. Claims 1 and 31-32 are rejected under 35 U.S.C. 103(a) as being unpatentable over by Wengel et al (PCT International Publication No. WO 99/14226, published 25 March 1999) in view of Beier et al (Science, vol. 283, pp. 699-703 (1989).

Regarding claims 31 and 32, the method of claim 31 is discussed above. Wengel et al do not teach alpha-L LNA monomers or xylo-LNA monomers,

However, Beier et al teach locked nucleic acids comprising alpha-L LNA monomers and xylo monomers (e.g., Scheme 1) with the added advantage that the monomers are stronger Watson-Crick binders (Abstract).

It would therefore have been obvious to a person of ordinary skill in the art at the time the invention was claimed to have modified to method as taught by Wengel et al with the monomers as taught by Beier et al with a reasonable expectation of success. The ordinary artisan would have been motivated to make such a modification because said modification would have resulted in stronger Watson-Crick binding as explicitly taught by Beier et al (Abstract).

5. Claims 1, 55, 56, 58, 59, 64-67, and 139 are rejected under 35 U.S.C. 103(a) as being unpatentable over by Wengel et al (PCT International Publication No. WO 99/14226, published 25 March 1999).

Regarding claim 55, the method of claim 1 is discussed above. Wengel et al also teach the LNA oligonucleotide is complementary to poly(A) tails in eukaryotic mRNA(e.g., poly dT probes [i.e., primers] containing LNA T residues are used to prime

Arabidopsis mRNA; page 181, Example 160) and where the said LNA oligonucleotide is synthesized with an anthraquinone moiety and a linker at the 5'-end of said oligonucleotide (e.g., the oligonucleotides carry an anthraquinone and a linker; page 62, lines 15-27), wherein said linker is a higher hexaethylene glycol polymer (e.g., polyethylene glycol; page 21, lines 10-15). While Wengel et al teach LNA oligonucleotides covalently coupled to a solid polymer support (e.g., LNA oligonucleotides are immobilized on a solid support; page 15, lines 7-9) via excitation of the anthraquinone moiety using UV light (page 62, lines 23-26 and page 166, Example 149), Wengel et al do not specifically teach immobilization of the poly dT primers.

However, Wengel et al do teach that immobilization of nucleic acids is preferred because it allows hybridization and capture to occur simultaneously (page 62, lines 4-10).

It would therefore have been obvious to a person of ordinary skill in the art at the time the invention was claimed to have modified the method of claim 1 as taught by Wengel et al by immobilizing the LNA molecules as also taught by Wengel with a reasonable expectation of success. The ordinary artisan would have been motivated to make the modification because the modification would have resulted in simultaneous hybridization and capture as explicitly taught by Wengel et al (page 62, lines 4-10).

Regarding claim 56, the method of claim 55 is discussed above. Wengel et al also teach detection with probes using chemiluminescence (e.g., an LNA nucleoside has a

reporter group [page 9, lines 1-19], wherein the reporter group uses chemiluminescence; page 19, lines 8-20)

Regarding claim 58, the method of claim 56 is discussed above. Wengel et al also teach LNA probes having several digoxigenin molecules (e.g., an LNA nucleoside has a reporter group [page 9, lines 1-19], wherein the reporter group is digoxigenin [page 19, lines 8-10], wherein a probe has multiple LNA nucleosides [e.g., Probe RTZ5, 5'-**TTTTTTTTTT**, where each LNA nucleotide is in bold; page 181], and the reporter groups is detected by antibodies to it; page 19, lines 22-25).

Regarding claim 59, the method of claim 55 is discussed above. Wengel et al also teach contacting the sample with a polymerase and at least one nucleotide (e.g., mRNA is subjected to reverse transcription; page 181, Example 160).

Regarding claim 64, the method of claim 59 is discussed above. Wengel et al also teach the polymerase comprises a reverse transcriptase (e.g., the MRNA is subjected to a Reverse Transcriptase reaction; page 181, Example 160).

Regarding claim 65 and 67, the method of claim 59 is discussed above. Wengel et al also teach labeled LNA oligonucleotides (e.g., an LNA nucleoside has a reporter group [page 9, lines 1-19], and wherein a probe has multiple LNA nucleosides; e.g., Probe RTZ5, 5'-**TTTTTTTTTT**, where each LNA nucleotide is in bold; page 181).

Regarding claim 66, the method of claim 59 is discussed above. Wengel et al also teach the LNA oligonucleotide is bound to a solid support (e.g., the MRNA is subjected

to a Reverse Transcriptase reaction and an amplification reaction [page 181, Example 160], and said reactions occur directly on a surface; page 62, lines 8-10).

Regarding claim 139, the method of claim 56 is discussed above. Wengel et al also teach digoxigenin incorporated into nucleic acid probes (e.g., an LNA nucleoside has a reporter group [page 9, lines 1-19], wherein the reporter group is digoxigenin; page 19, lines 8-10)

6. Claim 57 is rejected under 35 U.S.C. 103(a) as being unpatentable over by Wengel et al (PCT International Publication No. WO 99/14226, published 25 March 1999) as applied to claims 55 and 56 above, and further in view of Bobrow et al (U.S. Patent No. 5,731,158, issued 24 March 1998).

Regarding claim 57, the methods of claim 55-56 are discussed above. While Wengel et al teach reporter groups (page 9, lines 1-19), Wengel et al does not teach tyramide signal amplification.

However, Bobrow et al teach tyramide signal amplification (column 4, lines 36-49) as a reporter system for nucleic acid binding systems (column 6, lines 49-55) with the added advantage that the reporter system allows quantitation of the presence of the analyte (Abstract).

It would therefore have been obvious to a person of ordinary skill in the art at the time the invention was claimed to have modified the method using reporter groups as taught by Wengel et al with the tyramide signal amplification reporter group as

taught by Bobrow et al with a reasonable expectation of success. The ordinary artisan would have been motivated to make the modification because the modification would have resulted in allowing quantitation of the presence of the analyte as explicitly taught by Bobrow et al (Abstract).

7. Claims 60, 61, 69, 70, and 73 are rejected under 35 U.S.C. 103(a) as being unpatentable over by Wengel et al (PCT International Publication No. WO 99/14226, published 25 March 1999) as applied to claim 59 above, and further in view of Eberwine et al (U.S. Patent No. 5,514,545, issued 7 May 1996).

Regarding claim 60, the method of claim 59 is discussed above. Wengel et al are silent with respect to generating a plurality of copies of the eukaryotic mRNA.

However, Eberwine teaches a method of generating a plurality of copies of mRNA (e.g., amplification; column 4, lines 34-55) with the added advantage that amplification aids in the characterization of cell identity (Abstract).

It would therefore have been obvious to a person of ordinary skill in the art at the time the invention was claimed to have modified the method as taught by Wengel et al with amplification as taught Eberwine et al with a reasonable expectation of success. The ordinary artisan would have been motivated to make the modification because the modification would have resulted in aiding in the characterization of cell identity as explicitly taught by Eberwine (Abstract).

Regarding claim 61, the method of claim 60 is discussed above. Eberwine et al also teach a constant temperature (e.g., 4 hours at room temperature; Example 3).

Regarding claim 69, the method of claim 60 is discussed above. Wengel et al also teach the LNA oligonucleotide comprises a fluorophore moiety and a quencher moiety, positioned in such a way that a hybridized state of the oligonucleotide can be distinguished from an unbound state of the oligonucleotide by an increase in the fluorescent signal from the nucleotide (e.g., the LNA is a molecular beacon; page 64, lines 1-25).

Regarding claim 70, the method of claim 60 is discussed above. Wengel et al also teach detection of increased fluorescence of the molecular beacon (page 64, lines 1-25).

Regarding claim 73, the method of claim 59 is discussed above. Wenger et al do not teach adding a primer.

However, Eberwine et al tech a method of generating a plurality of copies of mRNA (e.g., amplification; column 4, lines 34-55) requiring primers (column 3, lines 1-10) with the added advantage that amplification aids in the characterization of cell identity (Abstract).

It would therefore have been obvious to a person of ordinary skill in the art at the time the invention was claimed to have modified the method as taught by Wengel et al with amplification using primers as taught Eberwine et al with a reasonable expectation of success. The ordinary artisan would have been motivated to make the

modification because the modification would have resulted in aiding in the characterization of cell identity as explicitly taught by Eberwine (Abstract).

8. Claims 59-60, 62-63, 68, and 72 are rejected under 35 U.S.C. 103(a) as being unpatentable over by Wengel et al (PCT International Publication No. WO 99/14226, published 25 March 1999) as applied to claim 59 above, in further view of Gruenert et al (U.S. Patent No. 5,804,383, issued 8 September 1998).

Regarding claim 59, the method of claim 55 is discussed above. While Wengel et al also teach the method of claim 59 as discussed above, an alternate interpretation of contacting the sample with a polymerase and at least one nucleotide is taught by Gruenert et al, which teaches a method of generating a plurality of copies of mRNA (e.g., making and amplifying a cDNA copy of the mRNA using reverse transcriptase polymerase chain reaction; i.e., RT-PCR; Abstract) with the added advantage of allowing analysis of gene expression of specific alleles (Abstract).

It would therefore have been obvious to a person of ordinary skill in the art at the time the invention was claimed to have modified the method as taught by Wengel et al to generate a plurality of copies of mRNA of as taught Gruenert et al with a reasonable expectation of success. The ordinary artisan would have been motivated to make the modification because the modification would have resulted in allowing analysis of gene expression of specific alleles as explicitly taught by Gruenert et al (Abstract).

Regarding claim 60, method of claim 59 is discussed above. Gruenert et al also teach generating a plurality of copies of mRNA (e.g., making and amplifying a cDNA copy of the mRNA using reverse transcriptase polymerase chain reaction; i.e., RT-PCR; Abstract) with the added advantage of allowing analysis of gene expression of specific alleles (Abstract).

Regarding claim 62, the method of claim 60 is discussed above. Gruenert et al also teach cycling the temperature of the sample (e.g., conducting PCR on the sample; Abstract).

Regarding claim 63, the method of claim 60 is discussed above. Gruenert et al also teach a thermally stable polymerase (e.g., Taq (Is) DNA polymerase; column 25, lines 24-25).

Regarding claim 68, the method of claim 59 is discussed above. Wengel et al do not teach cells stably associated with a solid support.

However, Gruenert et al teach a method of generating a plurality of copies of mRNA (e.g., making and amplifying a cDNA copy of the mRNA using reverse transcriptase polymerase chain reaction; Abstract) comprising nucleic acid molecules in cells wherein the cells are stably associated with a solid support (e.g., the nucleic acids for the RT-PCR are in cells fixed to a slide then submitted to RT-PCR; column 8, lines 31-60) with the added advantage that the procedure destroys proteins and enzymes that interfere with PCR (column 8, lines 31-50).

It would therefore have been obvious to a person of ordinary skill in the art at the time the invention was claimed to have modified the method as taught by Wengel et al to generate a plurality of copies of mRNA using cells stably associated with a solid support as taught Gruenert et al with a reasonable expectation of success. The ordinary artisan would have been motivated to make the modification because the modification would have resulted in destruction of proteins and enzymes that interfere with PCR as explicitly taught by Gruenert et al (column 8, lines 31-50).

Regarding claim 72, the method of claim 59 is discussed above. Gruenert et al also teach rTh polymerase (column 9, lines 34-50).

9. Claim 71 is rejected under 35 U.S.C. 103(a) as being unpatentable over by Wengel et al (PCT International Publication No. WO 99/14226, published 25 March 1999) and Eberwine et al (U.S. Patent No. 5,514,545, issued 7 May 1996) as applied to claim 70 above, further in view of Eis et al (Nature Biotechnology, vol. 9, pp. 673-676, (July 2001))

Regarding claim 71, the method of claim 70 is discussed above. Neither Wengel et al nor Eberwine teach cleavage of the LNA (i.e., cleavage of the probe that captures the RNA).

However, Eis et al teach a method of capturing mRNA using invasive cleavage (Figure 1), wherein a probe is cleaved from the mRNA (Figure 1) with the added advantage that invasive cleavage allows direct quantitation of specific RNAs (Title).

It would therefore have been obvious to a person of ordinary skill in the art at the time the invention was claimed to have modified the method as taught by Wengel et al with cleavage of the probe as taught Eis et al with a reasonable expectation of success. The ordinary artisan would have been motivated to make the modification because the modification would have resulted in allowing direct quantitation of specific RNAs as explicitly taught by Eis et al (Title).

10. Claims 77-80 and 140 are rejected under 35 U.S.C. 103(a) as being unpatentable over by Wengel et al (PCT International Publication No. WO 99/14226, published 25 March 1999) as applied to claims 59 above, in further view of Sambrook et al (*Molecular Cloning, A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, NY, pp. 5.58, 8.2, 8.3, 8.60-8.61, and 8.64-8.65 (1989)).

Regarding claim 77, the method of claim 59 is discussed above. Wengel et al do not teach adding a DNA polymerase.

However, Sambrook et al teach a method of amplifying mRNA comprising adding DNA polymerase, RNAse H (pages 8.60-8.61) and E. coli ligase after conversion of polyadenlylated mRNA to first strand complementary DNA under conditions suitable for generating double stranded complementary DNA (pages 8.64-8.65) with the added benefit of established a comprehensive cDNA library from a small quantity of mRNA (page 8.3, paragraph 1).

It would therefore have been obvious to a person of ordinary skill in the art at the time the invention was claimed to have modified the method as taught by Wengel et al with amplification using the steps as taught Sambrook et al with a reasonable expectation of success. The ordinary artisan would have been motivated to make the modification because the modification would have resulted in establishment of a comprehensive cDNA library from a small quantity of mRNA as explicitly taught by Sambrook et al (page 8.3, paragraph 1).

Regarding claim 78, the method of claim 77 is discussed above. Sambrook et al also teach insertion into a cloning vector (page 8.2).

Regarding claim 79, the method of claim 77 is discussed above. Sambrook et al also teach an anchor sequence for an RNA polymerase (e.g., a T7 RNA polymerase promoter; page 5.58, 5th paragraph).

Regarding claim 80, the method of claim 78 is discussed above. Sambrook et al also teach addition of T7 RNA polymerase to generate a plurality of RNA copies (page 5.58, first two paragraphs).

Regarding claim 140, the method of claim 79 is discussed above. Sambrook et al also teach T7 RNA polymerase (page 5.58, 5th paragraph).

11. Claims 1, 54, and 90 are rejected under 35 U.S.C. 103(a) as being unpatentable over by Wengel et al (PCT International Publication No. WO 99/14226, published 25 March 1999) in view of Gottschling et al (U.S. Patent No. 5,916,752, issued 29 June 1999).

Regarding claim 90, the method of claim 1 is discussed above. Wengel et al also teach the method of claim 54 wherein the LNA oligonucleotide hybridizes to complementary sequences of eukaryotic RNA (e.g., the LNA are used to activate genes of therapeutic interest [page 60, lines 21-24], wherein the therapeutic applications are in humans; page 18, lines 5-6). Wengel et al do not teach yeast RNA.

However, Gottschling et al teach hybridization using yeast RNA with the added advantage that yeast RNA sequences have substantial sequence homology to the human RNA sequences (e.g., telomerase, column 17, line 64-column 18, line 5) and that yeast is a genetically tractable organism directly applicable to mammalian cells (column 23, lines 55-61).

It would therefore have been obvious to a person of ordinary skill in the art at the time the invention was claimed to have modified the method as taught by Wengel et al with yeast RNA as taught by Gottschling et al with a reasonable expectation of success. The ordinary artisan would have been motivated to make the modification because the modification would have resulted in RNA sequences with substantial homology to human sequences in an as explicitly genetically tractable organism directly applicable to mammalian cells as explicitly taught by Gottschling et al (column 17, line 64-column 18, line 5 and column 23, lines 55-61).

12. Claims 92 and 93 are rejected under 35 U.S.C. 103(a) as being unpatentable over by Wengel et al (PCT International Publication No. WO 99/14226, published 25 March 1999) in view of Recker et al (U.S. Patent No. 5,691,153, issued 25 November 1997).

Regarding claim 93, Wengel et al teach the method of claim 92 for amplifying a target nucleic acid molecule the nucleotide sequence which is complementary to a LNA oligonucleotide capture probe, the method comprising: providing a sample containing nucleic acid molecules having repetitive base sequences (e.g., a sample with an sequence that binds to a first primer 5'-GGTGG**TTTG**TTTG-3', which contains the complement to the repetitive sequence GGTGGT; page 165, lines 10-15); and contacting the nucleic acid molecules from the sample with at least one LNA oligonucleotide capture probe to capture target nucleic acids (e.g., the LNA probe hybridizes to the targets; page 195, Example 148); and subjecting the captured nucleic acids to polymerase chain reaction, using primers to amplify the captured nucleic acid (e.g., the target is amplified in a PCR amplification using an additional primer in addition to the first primer [page 195, Example 148], and wherein captured nucleic acids are amplified directly on a surface; page 62, lines 4-10). Wengel et al are silent with respect to multiplex PCR.

However, Recker et al teach a method of screening nucleic acids (i.e., genome screening) using multiplex PCR with the added advantage that multiplex PCR increased the speed of throughput 10 fold (column 11, lines 20-32).

It would therefore have been obvious to a person of ordinary skill in the art at the time the invention was claimed to have modified the method comprising PCR as taught by Wengel et al with multiplex PCR as taught by Recker al with a reasonable expectation of success. The ordinary artisan would have been motivated to make the modification because the modification would have resulted in increasing the speed of throughput 10 fold as explicitly taught by Recker et al (column 11, lines 20-32).

13. Claims 110-113 are rejected under 35 U.S.C. 103(a) as being unpatentable over by Wengel et al (PCT International Publication No. WO 99/14226, published 25 March 1999) in view of Skouv et al (U.S. Patent No. 6,303,315 B1, issued 16 October 2001) and Alizon et al (U.S. Patent No. 5,310,651, issued 10 May 1994).

Regarding claim 110, Wengel et al teach a method for detecting an/or isolating a target nucleic acid molecule having a consecutively repeating base comprising: treating a sample containing nucleic acid molecules with an LNA oligonucleotide to thereby detect and/or isolate a nucleic acid molecule having said consecutively repeating base (e.g., poly dT primers containing LNA T residues are used to bind polyadenylated mRNA; page 181, Example 160). Wengel et al also teach LNA capture probes (e.g., LNA-modified oligonucleotides function efficiently in the sequence specific capture of RNA molecules; page 182, lines 23-24). Wengel et al do not teach chaotropic agents.

However, Skouv et al teach the preparation of nucleic acids from biological samples using LNA and lysing in the presence of chaotropic agents (Abstract)

concomitant with sample denaturation (column 47, lines 40-45) with the added advantage that chaotropic agents facilitate lysis of the cells (column 1, lines 38-42).

It would therefore have been obvious to a person of ordinary skill in the art at the time the invention was claimed to have modified to method as taught by Wengel et al with the chaotropic agent as taught by Skouv with a reasonable expectation of success. The ordinary artisan would have been motivated to make such a modification because said modification would have resulted in facilitation of the lysis of the cells as explicitly taught by Skouv (column 1, lines 38-42). Neither Wengel et al nor Skouv teach RNA genomes form infectious diseases.

However, Alizon et al teach the isolation of genomic RNA from an infectious disease causing organism (e.g., isolation of RNA from HIV; column 11, lines 1-22) having consecutively repeating nucleic basis (e.g., Figure 1B) with the added advantage that genomic RNA allows for diagnosis (Abstract).

It would therefore have been obvious to a person of ordinary skill in the art at the time the invention was claimed to have modified to method as taught by Wengel et al and Skouv by using genomic RNA from an infectious disease organism as taught by Alizon et al with a reasonable expectation of success. The ordinary artisan would have been motivated to make such a modification because said modification would have resulted in allowing diagnosis as explicitly taught by Alizon et al (Abstract).

Regarding claims 111-112, the method of claim 110 is discussed above. Skouv also teaches guanidinium thiocyanate at a concentration of at least about 4M (column 22, lines 1-8).

Regarding claim 113, the method of claim 110 is discussed above. Skouv also teaches a Tm of about 70 °C (column 10, lines 53-56).

14. Claims 114-119 and are rejected under 35 U.S.C. 103(a) as being unpatentable over by Wengel et al (PCT International Publication No. WO 99/14226, published 25 March 1999) and Skouv et al (U.S. Patent No. 6,303,315 B1, issued 16 October 2001) and Alizon et al (U.S. Patent No. 5,310,651, issued 10 May 1994) as applied to claim 110 above, and as defined by Sambrook et al *Molecular Cloning, A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, NY, page 7.5 (1989)).

Regarding claims 114-115, the method of claim 110 is discussed above. Skouv also teaches guanidinium thiocyanate at a concentration of at least about 4M (column 22, lines 1-8). Skouv also teaches beta-mercaptoethanol in the lysing and hybridizing buffer (Abstract and column 4, lines 58-67). Sambrook et al define 4M guanidinium thiocyanate and beta-mercaptoethanol as RNAse inhibitors (page 7.5). It is noted that *In re Best* (195 USPQ 430) and *In re Fitzgerald* (205 USPQ 594) discuss the support of rejections wherein the prior art discloses subject matter which there is reason to believe inherently includes functions that are newly cited or is identical to a product instantly claimed. In such a situation the burden is shifted to the applicants to "prove that

subject matter shown to be in the prior art does not posses characteristic relied on" (205 USPQ 594, second column, first full paragraph). The inclusion of 4M guanidinium thiocyanate and beta-mercaptoethanol in the lysing buffer taught by Skouv is therefore and RNAse inhibitor as required by the claims.

Regarding claims 116-117, the method of claim 114 is discussed above. Alizon et al also teach the genomic RNA is isolated from the retrovirus HIV (Abstract).

Regarding claim 118, the method of claim 114 is discussed above. Alizon et al also teach the genomic RNA is used to genotype RNA viruses (e.g., the method detects HIV-2 [Abstract], a specific genotype of HIV).

Regarding claim 119, the method of claim 114 is discussed above. Alizon et al also teach the genomic RNA is used for diagnosis in a patient (e.g., the RNA is isolated from infected patients to indicate if HIV-2 is present; column 5, lines 19-28).

15. Claims 136-138 are rejected under 35 U.S.C. 103(a) as being unpatentable over by Wengel et al (PCT International Publication No. WO 99/14226, published 25 March 1999) as applied to claim 56 above, and further in view of Squirrell et al (U.S. Patent No. 5,837,465, issued 17 November 1998).

Regarding claims 136-138, the method of claim 56 is discussed above. While Wengel et al also teach detection with probes using chemiluminescence (e.g., an LNA nucleoside has a reporter group [page 9, lines 1-19], wherein the reporter group uses

chemiluminescence; page 19, lines 8-20), Wengel does not specifically teach enzyme conjugated probes using chemiluminescence.

However, Baldwin et al teach nucleic acid probes with chemiluminescent enzymes (e.g. the bioluminescent enzyme luciferase; column 2, lines 14-26) with the added advantage that luciferase allows detection at very low concentrations using simple instruments (column 1, lines 15-20).

It would therefore have been obvious to a person of ordinary skill in the art at the time the invention was claimed to have modified to method as taught by Wengel et al by using luciferase as taught by Squirrell et al with a reasonable expectation of success. The ordinary artisan would have been motivated to make such a modification because said modification would have resulted in allowing detection at very low concentrations using simple instruments as explicitly taught by Squirrell et al (column 1, lines 15-20).

***Conclusion***

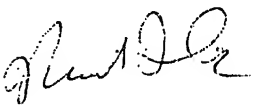
1. No claim is allowed.

2. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Robert T. Crow whose telephone number is (571) 272-1113. The examiner can normally be reached on Monday through Friday from 8:00 am to 4:30 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Ram Shukla can be reached on (571) 272-0735. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

Robert T. Crow
Examiner
Art Unit 1634



BJ FORMAN, PH.D.
PRIMARY EXAMINER

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|---|---|---|---|
| | Examiner Robert T. Crow | Art Unit 1634 | Page 1 of 1 |

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| | K | US- | | | |
| | L | US- | | | |
| | M | US- | | | |

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|---|---|---|---|---|---|---|
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*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
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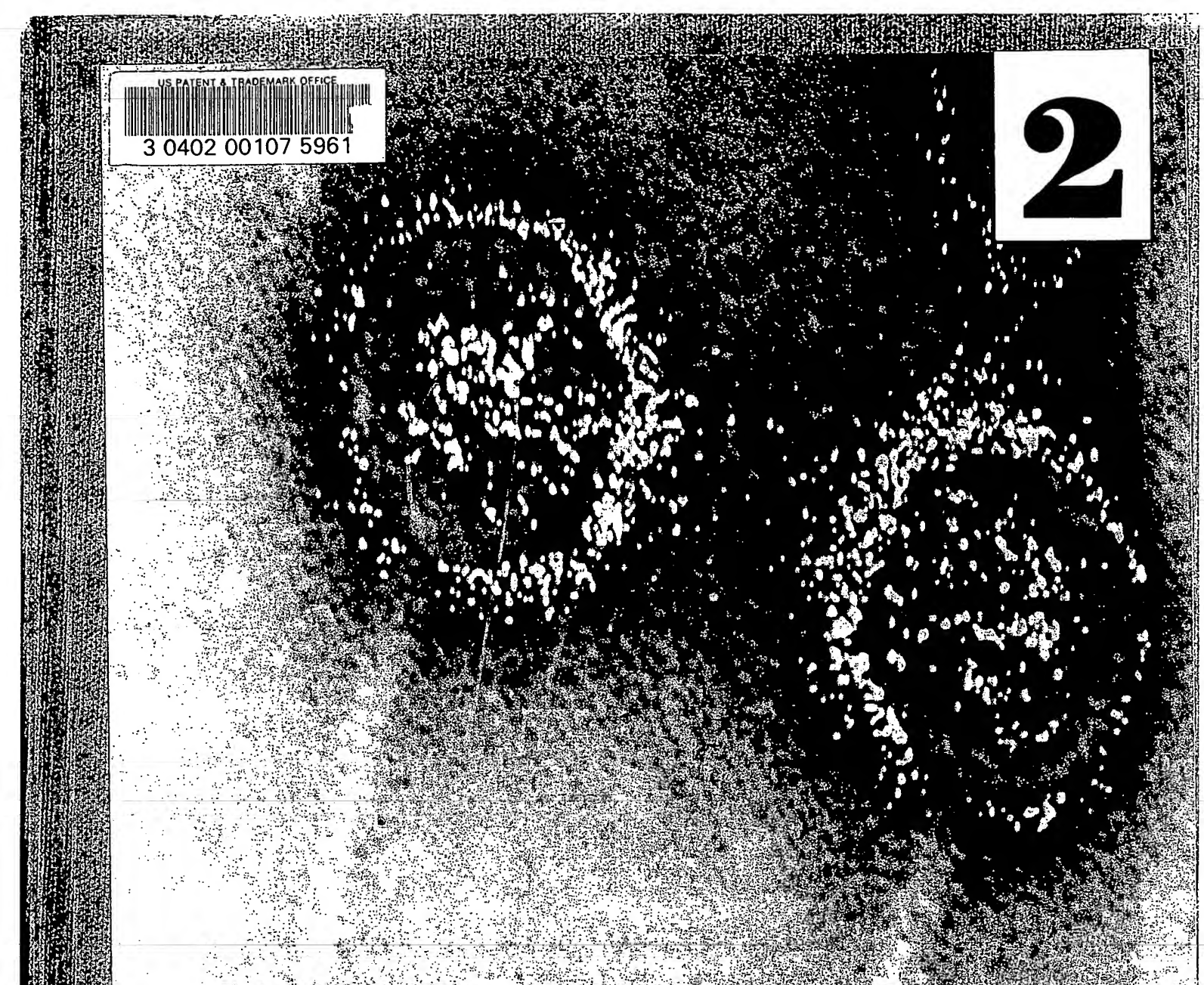

# Molecular Cloning

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## *DNA-DEPENDENT RNA POLYMERASES*

### *Bacteriophage SP6 and Bacteriophages T7 and T3 RNA Polymerases*

**(Bacteriophage SP6-infected *Salmonella typhimurium* LT2 and bacteriophage T7- or T3-infected *E. coli*)**

Bacteriophage SP6 synthesizes a DNA-dependent RNA polymerase that recognizes and initiates synthesis of RNA on double-stranded DNA templates that carry the appropriate bacteriophage-specific promoter. The polymerase is used in vitro to generate large quantities of RNA complementary to one strand of foreign DNA that has been cloned immediately downstream from the promoter in plasmids specifically designed for this purpose. Vectors are available to synthesize RNA complementary to either strand of the template by changing the orientation of the promoter with respect to the cloned foreign DNA sequences (Butler and Chamberlin 1982; Melton et al. 1984).

Bacteriophages T7 and T3 also synthesize DNA-dependent RNA polymerases that recognize and initiate synthesis of RNA on double-stranded DNA templates that carry the appropriate bacteriophage-specific promoter. These polymerases are used in vitro just like the bacteriophage SP6 RNA polymerase. Bacteriophages T7 and T3 RNA polymerases have been cloned and expressed in *E. coli* (Davanloo et al. 1984; Tabor and Richardson 1985; Morris et al. 1986) and bacteriophage T7 RNA polymerase has been cloned and expressed in yeast (Chen et al. 1987). This allows vectors carrying the bacteriophage T7 promoter to be used to express cloned genes in vivo.

**USES**

1. Synthesis of single-stranded RNA for use as hybridization probes, functional mRNAs for in vitro translation systems, or substrates for in vitro splicing reactions. Each of the three RNA polymerases has a high degree of specificity for its cognate promoter.

2. The bacteriophage T7 transcription system has been used to express cloned genes in bacteria (Tabor and Richardson 1985; Studier and Moffatt 1986) and in yeast (Chen et al. 1987).

   Two types of bacteriophage T7 expression systems have been developed for *E. coli*. In the first system, stable lysogens are established with bacteriophage λ carrying the bacteriophage T7 RNA polymerase gene under the control of the *E. coli lac*UV5 promoter. Plasmids containing the gene of interest under the control of the bacteriophage T7 promoter are then introduced into the lysogens containing the bacteriophage T7 RNA polymerase gene. Activation of the bacteriophage T7 promoter is then achieved by isopropylthio-β-D-galactoside induction of the *lac*UV5 promoter driving the bacteriophage T7 RNA polymerase gene. In the second system, the bacteriophage T7 promoter/plasmid carrying the gene of interest is introduced into bacteria, and the bacteriophage T7 promoter is activated by infecting the bacteria with bacteriophage λ containing the bacteriophage T7 RNA polymerase gene.

   In yeast, the bacteriophage T7 RNA polymerase gene is placed under the control of a yeast promoter and stably introduced into yeast cells on an autonomously replicating vector. Expression is achieved by introducing into the yeast cells a second plasmid that contains the gene of interest under the control of the bacteriophage T7 promoter (Chen et al. 1987).

and can be used as a template in some in vitro enzymatic reactions (e.g., reverse transcription of mRNA). However, vanadyl-ribonucleoside complexes strongly inhibit translation of mRNA in cell-free systems and must be removed from the mRNA by multiple extractions with phenol (equilibrated with 0.01 M Tris · Cl [pH 7.8]) containing 0.1% hydroxyquinoline. Vanadyl-ribonucleoside complexes are available from several commercial suppliers.

- *Macaloid.* Macaloid is a clay that has been known for many years to adsorb RNAase. The clay is prepared as a slurry (see Appendix B) that is used at a final concentration of 0.015% (w/v) in buffers used to lyse cells (Favaloro et al. 1980). The clay, together with its adsorbed RNAase, is removed by centrifugation at some stage during the subsequent purification of the RNA (e.g., after extraction with phenol).

### ***hods That Disrupt Cells and Inactivate Ribonucleases ultaneously***

Proteins dissolve readily in solutions of potent denaturing agents such as guanidine HCl and guanidinium thiocyanate (Cox 1968). Cellular structures disintegrate and nucleoproteins dissociate from nucleic acids as protein secondary structure is lost. RNAases can recover activity after many forms of treatment (such as boiling) but are inactivated by 4 M guanidinium thiocyanate and reducing agents such as $\beta$-mercaptoethanol (Sela et al. 1957). This combination of reagents can therefore be used to isolate intact RNA from tissues, such as the pancreas, that are rich in RNAase (Chirgwin et al. 1979).

The protocols presented below use inhibitors of RNAase and/or methods that lead to the rapid inactivation of RNAases for the isolation of total, nuclear, and cytoplasmic RNAs from tissues and cultured cells.

The process of enzymatic conversion of poly(A)$^+$ mRNA to double-stranded DNA and the insertion of this DNA into prokaryotic vectors has become a fundamental tool of eukaryotic molecular biology. Since the first clones of complementary DNA (cDNA) were obtained in the mid-1970s, many different methods have been developed to increase the efficiency of synthesis of double-stranded cDNA and many improvements have been made to the vector systems. In the first part of this chapter, we describe some of these developments, pointing out their advantages and disadvantages. In the second part, we present in detail the procedure that is now most commonly used: synthesis of the first strand of cDNA with reverse transcriptase, replacement synthesis of the second strand of cDNA (using RNAase H and the Klenow fragment of *Escherichia coli* DNA polymerase I), addition of synthetic linkers, and finally, cloning into a bacteriophage $\lambda$ vector.

# Strategies for cDNA Cloning

The synthesis and cloning of cDNA are still not easy, but as a consequence of a wide range of technical and theoretical advances, cDNA cloning is now well within the range of any competent laboratory. Comprehensive cDNA libraries can be routinely established from small quantities of mRNA, and a variety of reliable methods are available to identify cDNA clones corresponding to extremely rare species of mRNA. As the enzymatic reactions used to synthesize cDNA have improved, the sizes of cloned cDNAs have increased, and it is often possible to isolate cloned full-length cDNAs corresponding to large mRNAs.

Before embarking on the synthesis and cloning of cDNA, however, it is essential to consider carefully which methods, vectors, and screening procedures offer the best chance of success. Which combination of these factors is most appropriate for a particular cloning problem depends on the information and reagents that are available, for example, whether the sequence of the protein is known or can be obtained or whether suitable antibodies are available.

## PREPARATION OF mRNA FOR cDNA CLONING

### Source of the mRNA

Clearly, the higher the concentration of the sequences of interest in the starting mRNA, the easier the task of isolating relevant cDNA clones becomes. It is therefore worthwhile investing some effort to make sure that the richest source of mRNA available is being used. This can be achieved, for example, by using immunoprecipitation to measure the amount of the protein of interest that is synthesized in cell-free systems by mRNAs prepared from different cell lines or tissues. In addition, some workers have found ways to increase the concentration of the relevant mRNA by using drugs to select cell lines that overexpress particular proteins. For example, Luskey et al. (1983) describe the use of the drug compactin to develop a line of Chinese hamster ovary cells that have undergone a 15-fold increase in the number of gene copies and a much greater increase in the amount of mRNA coding for 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase). Other workers have taken advantage of the observation that treatment of infected cells with inhibitors of protein synthesis causes extended transcription of the early genes of mammalian DNA viruses (e.g., Persson et al. 1981). In these and a number of similar cases, increased abundance of specific classes of rare mRNAs greatly facilitated their subsequent cloning.

Whenever possible, estimates should be obtained of the frequency with which the mRNA of interest occurs in the starting preparation. As discussed below, this frequency not only determines the size of the cDNA library that is needed, but it may also influence the method used to screen it.

### *Protocol for the Synthesis of the First Strand of cDNA*

**1.** Mix in a sterile microfuge tube on ice:

| | |
|---|---|
| poly(A)$^+$ RNA (1 mg/ml) | 10.0 $\mu$l |
| oligo(dT)$_{12-18}$ (1 mg/ml) | 10.0 $\mu$l |
| 1 M Tris · Cl (pH 7.6) | 2.5 $\mu$l |
| 1 M KCl | 3.5 $\mu$l |
| 250 mM $MgCl_2$ | 2.0 $\mu$l |
| mixture of dNTPs (5 mM each) | 10.0 $\mu$l |
| 0.1 M dithiothreitol | 2.0 $\mu$l |
| RNAase inhibitor | 25 units |
| $H_2O$ to a final volume of 48 $\mu$l | |

Add 2 $\mu$l of murine reverse transcriptase (Pharmacia, 15,000–30,000 units/ml; for other manufacturers [see Table 8.1], use an equivalent number of units). Mix well by gentle vortexing. Try to avoid producing bubbles, although this is sometimes difficult because reverse transcriptase is supplied in a buffer containing Triton X-100. If necessary, bubbles may be removed by brief centrifugation in a microfuge.

No radioactive tracer is included in the large-scale first-strand reaction described above. To analyze the material produced in the reaction, it is necessary to carry out a small-scale parallel reaction:

a. After all components of the large-scale reaction have been mixed at 4°C, place 2.5 $\mu$l of the reaction in a fresh, small (0.5-ml) microfuge tube.

b. Add 0.1 $\mu$l of [$\alpha$-$^{32}$P]dCTP (400 Ci/mmole, 10 $\mu$Ci/$\mu$l) to the small-scale reaction.

c. Incubate both reactions for 1 hour at 37°C.

d. Count the total and TCA-precipitable radioactivities in 0.5 $\mu$l of the small-scale reaction. Store the large-scale reaction at 4°C.

e. Store the remainder of the small-scale reaction at −20°C for later analysis by electrophoresis through an alkaline agarose gel (see Chapter 6, pages 6.20–6.21).

If you are using avian reverse transcriptase, the buffer should be altered appropriately (see Note iii).

**2.** Calculate the amount of first strand synthesized as follows:

a. Since 10 $\mu$l of a solution containing all four dNTPs at a concentration of 5 mM each was used (i.e., 10 $\mu$l of 20 mmoles/liter of total dNTP), the large-scale first-strand reaction therefore contains

$$20 \text{ nmoles}/\mu\text{l dNTP} \times 10\ \mu\text{l} = 200 \text{ nmoles of dNTP}$$

b. Because the molecular weight of each dNTP incorporated into DNA is approximately 330, the reaction is capable of generating a total of

$$200 \text{ nmoles} \times 330 \text{ ng/nmole} = 66\ \mu\text{g of DNA}$$

c. Therefore, from the results of the small-scale reaction,

$$\frac{\text{cpm incorporated}}{\text{total cpm}} \times 66\ \mu g = \mu g \text{ of first strand of cDNA synthesized}$$

The yield of first strand is usually close to 50% of the weight of the poly(A)$^+$ RNA in the reaction mixture.

**Notes**

i. An alternative procedure to monitor synthesis of the first strand of cDNA is to use a small amount of [$\alpha$-$^{32}$P]dCTP (10 $\mu$Ci total) in the large-scale reaction described above and a much greater amount (100 $\mu$Ci total) of radiolabeled dNTP in the subsequent reaction to synthesize the second strand of cDNA. This procedure is less desirable because it is then necessary to expose the alkaline agarose gel to X-ray film for long periods of time to obtain a suitable image of the first-strand products.

ii. If enough poly(A)$^+$ RNA is available, it is advisable to determine empirically the amount of reverse transcriptase that results in the highest yields and longest cDNAs. Small-scale reactions (each containing 0.5–1.0 $\mu$g of poly(A)$^+$ RNA) should be set up using the conditions described below and including varying amounts of reverse transcriptase (10–200 units/$\mu$g of RNA). The yield of cDNA should be determined by precipitation with ice-cold TCA (see Appendix E), and its size should be determined by electrophoresis through alkaline agarose gels (see Chapter 6, pages 6.20–6.21). For large-scale synthesis of cDNA, use the concentration of enzyme that gives the greatest yield of long cDNA. If limiting amounts of poly(A)$^+$ RNA are available, use 40–50 units of reverse transcriptase per microgram of RNA.

*Pilot reactions for synthesis of the first strand of cDNA*

a. Mix one or more pilot reactions on ice in sterile microfuge tubes:

| | |
|---|---|
| poly(A)$^+$ RNA (0.5–1.0 mg/ml) | 1.0 $\mu$l |
| oligo(dT)$_{12-18}$ (1 mg/ml) | 2.0 $\mu$l |
| 1 M Tris · Cl (pH 7.6) | 1.5 $\mu$l |
| 1 M KCl | 1.5 $\mu$l |
| 250 mM $MgCl_2$ | 1.0 $\mu$l |
| mixture of dNTPs (5 mM each) | 5.0 $\mu$l |
| 0.1 M dithiothreitol | 1.0 $\mu$l |
| RNAase inhibitor | 10 units |
| [$\alpha$-$^{32}$P]dCTP (400 Ci/mmole; 10 $\mu$Ci/$\mu$l) | 1.0 $\mu$l |
| $H_2O$ to a final volume of 25 $\mu$l | |

Add to each assay varying amounts of murine reverse transcriptase (10–200 units) per microgram of mRNA. Mix well by gentle vortexing. Remove bubbles by brief centrifugation in a microfuge.

Incubate for 2 hours at 37°C.

If you are using avian reverse transcriptase, the buffer for the reaction should be altered appropriately (see Note iii).

### *Protocol for the Synthesis of the Second Strand of cDNA*

The unlabeled first-strand reaction needs no further treatment before proceeding to the synthesis of the second strand of cDNA.

1. Add the following reagents directly to the first-strand reaction mixture:

| | |
|---|---|
| 10 mM $MgCl_2$ | 70.0 $\mu$l |
| 2 M Tris · Cl (pH 7.4) | 5.0 $\mu$l |
| [$\alpha$-$^{32}$P]dCTP (400 Ci/mmole; 10 $\mu$Ci/$\mu$l) | 10.0 $\mu$l |
| 1 M $(NH_4)_2SO_4$ | 1.5 $\mu$l |
| RNAase H (1000 units/ml) | 1.0 $\mu$l |
| *E. coli* DNA polymerase I (10,000 units/ml) | 4.5 $\mu$l |

Incubate for 4 hours at 16°C.

2. Add:

| | |
|---|---|
| 50 mM NAD | 1.0 $\mu$l |
| *E. coli* DNA ligase (100,000 units/ml) | 1.0 $\mu$l |
| bacteriophage T4 polynucleotide kinase (3000 units/ml) | 1.0 $\mu$l |

Incubate for 15 minutes at room temperature. *E. coli* DNA ligase will repair nicks in the DNA and the kinase will phosphorylate the 5′-hydroxyl group of the oligo(dT) primers.

3. Add 5 $\mu$l of 0.5 M EDTA (pH 8.0) to stop the reaction. Remove a small aliquot (3 $\mu$l) and store the remainder of the sample at −20°C until needed in step 6. Count the total and TCA-precipitable radioactivities in 0.5 $\mu$l of the small aliquot.

4. Calculate the weight of the cDNA synthesized in the second-strand reaction, taking into account the amount of dNTPs already incorporated into the first strand of cDNA:

$$\frac{\text{cpm incorporated in the second-strand reaction}}{\text{total cpm}} \times (66\ \mu g - x\ \mu g)$$
$$= \mu g \text{ of the second strand of cDNA synthesized}$$

where $x$ = weight of the first strand of cDNA. The amount of second-strand cDNA synthesized is usually 70–80% of the weight of the first strand.

5. Analyze the sizes of the radiolabeled first- and second-strand products by electrophoresis through an alkaline 1% agarose gel (see Chapter 6, pages 6.20–6.21), using end-labeled fragments of a *Hin*dIII digest of wild-type bacteriophage λ DNA as markers (see Chapter 10). If the reactions have worked well, the first strand of cDNA should exhibit a broad distribution of sizes (300 bases to more than 5 kb), with the majority of the material ranging between 1 and 2 kb.

It is very important that the size of the second strand of cDNA be identical to that of the first. A detectable increase in size indicates that a significant proportion of the

second-strand molecules have been generated by self-priming rather than by replacement synthesis. This can be verified as follows:

a. Treat two aliquots of radiolabeled first strand and two aliquots of radiolabeled second strand with 1 mM EDTA (pH 8.0), 300 mN NaOH for 30 minutes at 60°C. This hydrolyzes the RNA and denatures the DNA in the samples. Each aliquot should contain at least $10^4$ cpm.
b. Add 0.1 volume of 3 N HCl, 0.1 M Tris · Cl (pH 7.4) to each aliquot.
c. Purify the DNAs by extraction with phenol:chloroform and precipitation with ethanol.
d. Redissolve the DNAs in 10 μl of $H_2O$. Add 1 μl of 3 M NaCl. Incubate the DNAs for 5 minutes at 68°C to allow "hairpins" to form in the DNAs.
e. Add 20 μl of $H_2O$ and 3 μl of 10 × nuclease-S1 buffer.

*10 × Nuclease-S1 buffer*
2 M NaCl
0.5 M sodium acetate (pH 4.5)
10 mM $ZnSO_4$
5% glycerol

f. Add 5 units of nuclease S1 to one of the two tubes containing the first strand of cDNA and to one containing the second strand of cDNA. Incubate all four tubes for 30 minutes at 37°C.
g. Analyze the size of first- and second-strand products by electrophoresis through an alkaline 1% agarose gel (see Chapter 6, pages 6.20–6.21), using end-labeled fragments of a *Hin*dIII digest of wild-type bacteriophage λ DNA as markers (see Chapter 10).

*Note:* The first-strand molecules should be completely hydrolyzed by nuclease S1, and no more than 10–15% of the second-strand molecules should be resistant to digestion with the enzyme. If the proportion of self-primed molecules is unacceptably high, it is often helpful to adjust the amount of RNAase H used during the synthesis of the second strand. In addition, self-priming may be suppressed by including actinomycin D (50 μg/ml) or 4 mM sodium pyrophosphate (Rhyner et al. 1986) in first-strand reactions catalyzed by avian reverse transcriptase. Sodium pyrophosphate inhibits the activity of the murine enzyme (Roth et al. 1985).

**Caution:** Actinomycin D is a teratogen and carcinogen. Stock solutions should be prepared, wearing gloves and a mask, in a chemical hood, not on an open bench.

**6.** Thaw the remainder of the sample (stored in step 3) and extract with an equal volume of phenol:chloroform. Separate the unincorporated dNTPs from the cDNA by spun-column chromatography through Sephadex G-50 equilibrated in TE (pH 7.6) containing 10 mM NaCl (see Appendix E).

**7.** Precipitate the eluted cDNA by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol. Store the sample at room temperature for at least 15 minutes. Recover the precipitated DNA by centrifugation at 12,000*g* for 15 minutes at 4°C in a microfuge. Use a hand-held minimonitor to check that all of the radioactivity has been precipitated. Wash the pellet with 70% ethanol and recentrifuge. Gently aspirate all of the fluid (check to see that none of the radioactivity is in the aspirated fluid), and allow the pellet to dry in the air.

**8.** Redissolve the DNA in 80 μl of TE (pH 7.6) if it is to be methylated by M.*Eco*RI methylase (see below) or in 29 μl of TE (pH 7.6) if it is to be ligated directly to *Not*I or *Sal*I linkers (see page 8.68).

18. Response latencies were given by the time of the first significant deviation of the derivative of the low-pass filtered (5-ms wide gaussian) averaged membrane potential waveform ($t$ test, $P < 0.005$). The measure was validated when the peak of the rising waveform was significantly higher than that measured during the prestimulus trigger condition ($t$ test, $P < 0.01$). Latency profiles were significantly (least-mean-square optimization) fitted by two straight lines (average $r^2 = 0.77$ with 2D impulse-like input and 0.88 with long bars), the intersection of which defined the retinal position of the latency basin center (Fig. 4, B and C). This center was superimposed on or close to the MDF center (relative eccentricity: 0.85 ± 0.7°).
19. Despite the fact that both the latency and the strength of the response were highly correlated with the relative eccentricity from the MDF center, both features of the postsynaptic response were weakly correlated together, thus reinforcing the hypothesis that the causative independent variable is the relative eccentricity itself.
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21. A similar range of ASHP values is found in the adult cat if one takes into account the dependency of the cortical magnification factor on retinal eccentricity [K. Albus, *Exp. Brain Res.* **24**, 181 (1975); R. J. Tusa *et al.*, *J. Comp. Neurol.* **177**, 213 (1978)]. Furthermore, the ASHP values derived from individual latency basins are not significantly linked to the absolute retinal eccentricity of the discharge field of the recorded cells.
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24. The typical speed of action potential propagation along horizontal excitatory connections is 0.15 to 0.60 m/s in T. Murakoshi *et al.* [*Neurosci. Lett.* **163**, 211 (1993)], 0.16 m/s in D. A. Nelson and L. C. Katz [*Neuron* **15**, 23 (1995)], and 0.35 m/s in J. A. Hirsch and C. D. Gilbert [*J. Neurosci.* **11**, 1800 (1991)]. A similar estimate is found for inhibitory connections: 0.06 to 0.20 m/s in P. Salin and D. A. Prince [*J. Neurophysiol.* **75**, 1589 (1996)].
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29. We thank P. Godement, K. Grant, and P.-M. Lledo for helpful comments. We are indebted to L. Borg-Graham and C. Monier for patch electrode data, to D. E. Shulz and V. Ego for LGN recordings, and to P. Baudot, who participated in some of the experiments. This work was supported by CNRS, PROGRES-INSERM, AFIRST, GIS-Cognisciences, and Human Frontier Science Program (RG0103/1998-B) grants to Y.F.

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# Chemical Etiology of Nucleic Acid Structure: Comparing Pentopyranosyl-(2′→4′) Oligonucleotides with RNA

**M. Beier, F. Reck, T. Wagner, R. Krishnamurthy, A. Eschenmoser***

All four members of the family of pentopyranosyl-(2′→4′) oligonucleotide systems that contain β-ribo-, β-xylo-, α-lyxo-, or α-arabinopyranosyl units as repeating sugar building blocks are found to be much stronger Watson-Crick base-pairing systems than RNA. The α-arabinopyranosyl system is the strongest of all and in fact belongs to the strongest oligonucleotide base-pairing systems known. Whatever the chemical determinants by which nature selected RNA as a genetic system, maximization of base-pairing strengths within the domain of pentose-derived oligonucleotide systems was not the critical selection criterion.

A chemical understanding of the criteria by which nature chose ribo- and deoxyribonucleic acids as genetic systems would constitute a central element of any theory of the origin of the particular kind of chemical life that we know today. The quest for such an understanding may be taken up by experiment by systematically synthesizing potential alternatives to the natural nucleic acids and comparing them with RNA with respect to those chemical properties that are fundamental to RNA's biological function (*1*). For such an alternative to be selected for study, we require it to be structurally derivable from a $(CH_2O)_n$ aldosugar ($n$ = 6, 5, or 4) by the same type of potentially natural chemistry that allows the structure of RNA to be derived from ribose (*2*). This strategy is an attempt to mimic a hypothetical natural process that may have led to the selection of RNA: a process of combinatorial molecular assembly and functional selection within the domain of sugar-based oligonucleotides. In principle, such an experimental etiological analysis of nucleic acid structure is unbiased with respect to the question of whether RNA first came into being abiotically or biotically.

Our previous studies involving the β-hexopyranosyl-(4′→6′) oligonucleotide family had shown that base pairing in allo-, altro-, and glucopyranosyl oligonucleotides is uniformly much weaker than in RNA (*3*). Comparison with the properties of the 2′-deoxy, 3′-deoxy, and 2′,3′-dideoxyallopyranosyl analogs (*4*) demonstrated that the weaker base pairing is due to intrastrand steric hindrance in the pairing conformation ("too many atoms") (*3*). These findings led us to refocus our studies on the less bulky pentopyranosyl series, where it was discovered that the β-ribopyranosyl-(2′→4′) oligonucleotide system, the pyranosyl isomer of RNA (p-RNA), exhibits Watson-Crick pairing that is far stronger than that in RNA (*5*). Here we show that the same is true for the entire family of pentopyranosyl-(2′→4′) oligonucleotide systems that have the nucleotide base in the equatorial position of the pyranose chair (Scheme 1).

Scheme 2 summarizes the syntheses of the phosphoramidite building blocks **4**, **9**, and **13**, each prepared from the corresponding nucleosides containing adenine or thymine as the nucleobase (*6*, *7*). The preparation of oligomers in the lyxo- and xylopyranosyl series followed the (2′→4′) strategy previously applied in the p-RNA series (*5*). Inversely, oligomer synthesis in the α-arabinopyranosyl series was chosen to proceed in the (4′→2′) direction because the axial 4′-hydroxyl is the least reactive to electrophilic derivatization among the three hydroxyl groups (*8*).

Table 1 summarizes $T_m$ values (the temperature at which about 50% of duplex molecules are dissociated into single strands) and thermodynamic data for five different octamer duplexes of each of the four pentopyranosyl-(2′→4′) oligonucleotide systems, determined in buffered 0.15 M sodium chloride solution at pH 7.0 (see also Fig. 1). Duplex formation was further characterized by temperature-dependent circular dichroism (CD) spectroscopy (Fig. 2) as well as by confirmation of strand stochiometry by determination of ultraviolet (UV) mixing curves

The Skaggs Institute for Chemical Biology at The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA, and Laboratorium für Organische Chemie, Eidgenössische Technische Hochschule (ETH), Universitätstrasse 16, CH-8092 Zürich, Switzerland.

*To whom correspondence should be addressed.

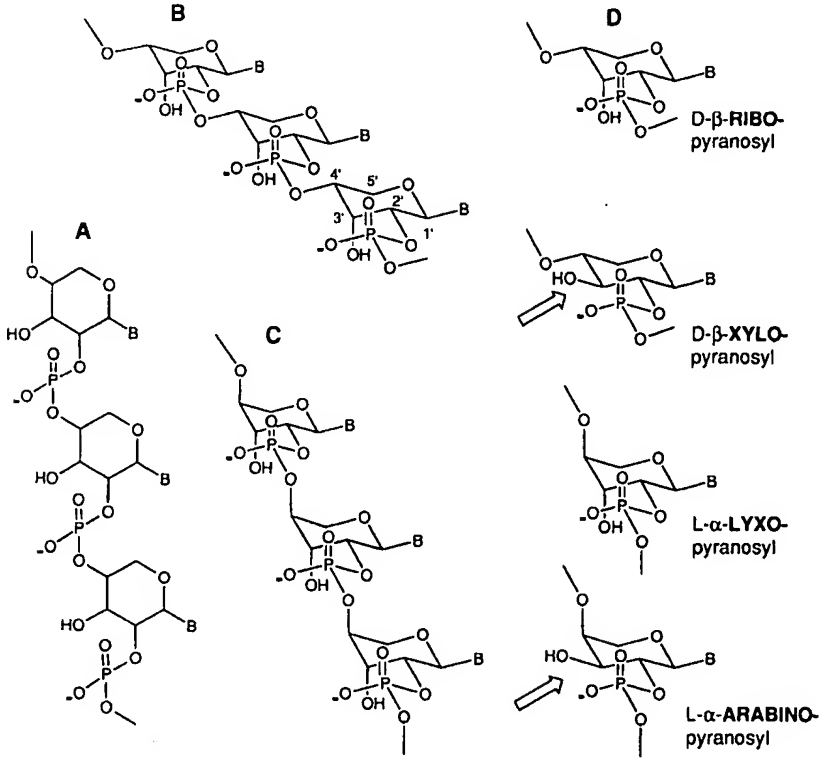
for the $A_8$·$T_8$ duplexes. There is no self-pairing of single strands in the xylopyranosyl series, whereas in the more strongly pairing α-lyxopyranosyl system, both $A_8$ and $T_8$ exhibit weak self-pairing under high-salt conditions. In the arabinopyranosyl series, $T_8$ pairs weakly with itself, whereas $A_8$ does not (*9*).

All four members of the pentopyranosyl-(2′→4′) oligonucleotide family undergo markedly efficient cross-pairing with each other, at least with regard to duplex formation between $A_8$ and $T_8$ strands (*10*). Such a cross-pairing must reflect the capability of all members to adopt a common type of duplex structure. We view it as consisting of quasi-linear Watson-Crick paired double strands with antiparallel strand orientation and a left-handed helical twist, as previously postulated for the $(CGAATTCG)_2$ duplex in the ribopyranosyl series on the basis of nuclear magnetic resonance (NMR) structural analysis and molecular dynamics modeling (*5*). The structural similarity among the duplexes is also reflected in their common characteristics with respect to the sequence dependence of their stability. Sequence motifs of the form $(pyrimidine)_n$-$(purine)_n$ are more stable than those of the form $(purine)_n$-$(pyrimidine)_n$ in all four systems of the pentopyranosyl-(2′→4′) oligonucleotide family (Fig. 1). Such behavior was previously rationalized in the p-RNA series (*5*) as being the consequence of the overriding influence of interstrand base-stacking on duplex stability in oligonucleotide systems that show a strong inclination between backbone and base-pair axes. This interpretation is supported by the inverse sequence-motif dependence of duplex stability in the homo-DNA series where the sign of backbone–base pair inclination is opposite to that of p-RNA (*4*, *11*).

Base pairing in p-RNA had been shown to be Watson-Crick by NMR spectroscopy (*5*), and we conclude that the same holds for the entire pentopyranosyl family. Supporting this conclusion is the observation that the sequence dependence of duplex stabilities follows a similar pattern for each of the four pentopyranosyl systems (Fig. 1) (*12*). The CD spectra of β-xylo- and β-ribopyranosyl duplexes closely resemble each other, as do the spectra of α-lyxo- and α-arabinopyranosyl duplexes (Fig. 2).

The α-arabinopyranosyl system, as judged from its $T_m$ values of adenine- and thymine-containing duplexes, belongs to the strongest phosphodiester-based oligonucleotide base-pairing systems encountered thus far (*13*). Its extraordinary pairing strength must be a consequence of the extensive steric constraint exerted on the phosphodiester group when flanked at the 2′-junction not only by the nucleobase, but also by the 3′-hydroxyl in an equatorial position (see arrows in Scheme 1); this constraint—in conjunction with the rigidity of the pyranose chair and the axial junction of the phosphodiester group at the 4′-carbon—may preorganize the single strand toward a pairing conformation (*4*). The phenomenon clearly calls for a detailed structural study (*14*). Because in the p-RNA series (*5*) the assessment of pairing behavior based on adenine-thymine pairing was found to be fully consistent with that derived from the behavior of guanine-cytosine–containing duplexes, we assume the same to be the case for all four pentopyranosyl-(2′→4′) oligonucleotide systems.

The finding that an entire family of potentially natural RNA isomers has a capability for Watson-Crick base pairing far more efficient than that of natural RNA itself needs to be put into an etiological perspective. From our present, although limited, knowledge of the range and type of chemistry that may have been involved in a primordial emergence of the RNA structure (*15*), we conjecture that such chemistry should have produced oligonucleotides derived not only from ribose, but from other aldopentoses as well, and that it might have led to both the furanosyl systems and the corresponding pyranosyl




Scheme 1. (A) Constitution of the pentopyranosyl-(2′→4′) oligonucleotide family. B = nucleotide base. (B) Idealized pairing conformation in duplexes of (D)-β-ribopyranosyl-(2′→4′) oligonucleotides. (C) Idealized pairing conformation in duplexes of (L)-α-lyxopyranosyl-(2′→4′) oligonucleotides. (D) Configuration and (idealized) conformation of the repeating units of the four diastereomeric pentopyranosyl-(2′→4′) oligonucleotide systems discussed in this paper. Arrows (⇒) point to steric hindrance between equatorial hydroxyl and equatorial phosphate groups (equatorial substituents lie in averaged plane of pyranosyl chair, axial substituents perpendicular to it).

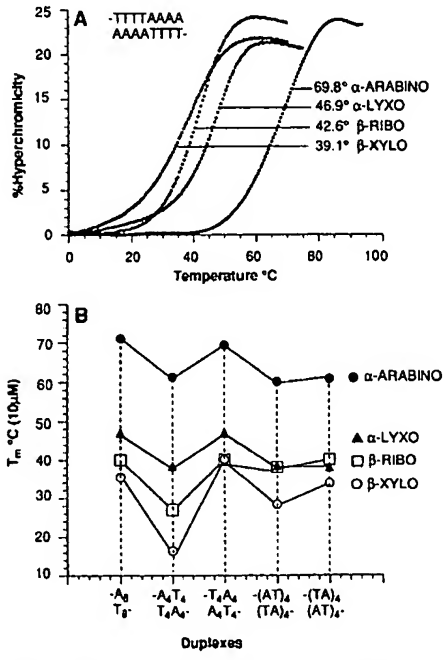


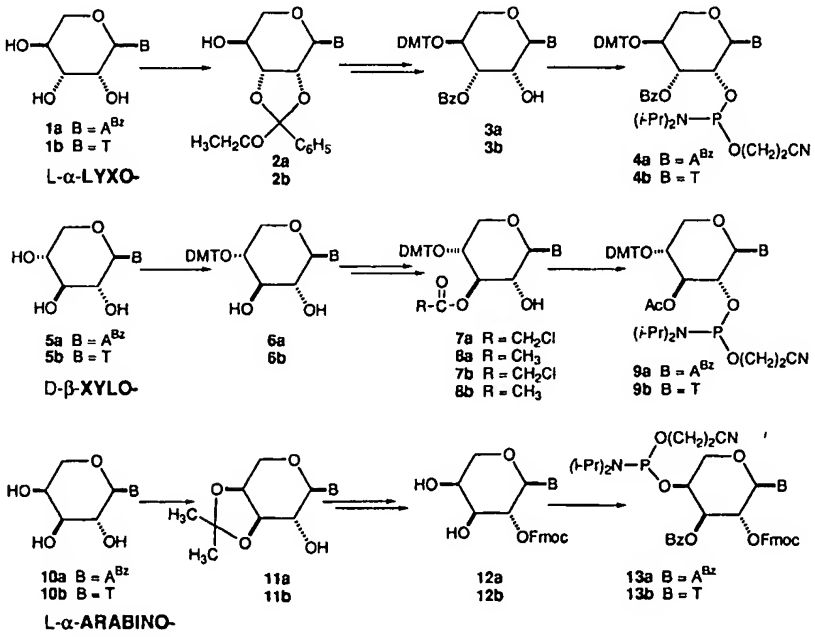

Fig. 1. (A) UV melting curves and $T_m$ values of self-complementary strands $T_4A_4$ in the four pentopyranosyl-(2′→4′) oligonucleotide systems [strand concentration $c \sim 10$ μM, 0.15 M NaCl, 10 mM $NaH_2PO_4$, 0.1 mM $Na_2EDTA$ (pH 7)]. (B) Thermal stabilities of octamer duplexes showing the pattern of sequence-motif dependence (for conditions see Table 1).

alternatives (*1*, *3*). In such a scenario, RNA eventually would have emerged from a library of structural alternatives by selection processes based on functional criteria. It is primarily with respect to this type of scenario that our findings are interpreted to demonstrate that whatever the decisive criterion responsible for nature's selection of RNA as a genetic system may have been, it was not the criterion of maximization of base-pairing strength. On the other hand, our observations substantiate the notion that it may have been optimization rather than maximization of the strength of base pairing that played a decisive role in RNA's emergence. Extension of the experimental approach to this question would call for an analysis of etiologically more demanding properties such as the capability of informational self-replication and the capacity to express a chemical phenotype (*16*). Preliminary information in this direction is available for p-RNA (*5*).

It is also conceivable that RNA entered the biological scene as a result of what would amount to a "frozen accident" in the sense that circumstances happened to favor a formation of the RNA structure in preference over alternatives. In that case, a synthetic rather than a functional selection would have been the primary determinant of RNA's emergence. Known chemical properties that could have facilitated such a synthetic selection include the intrinsic preference for ribose formation in kinetically (but not thermodynamically) controlled aldolizations (*17*), or the quenching of the furanose-pyranose equilibrium of ribose in favor of the furanose form by selective functionalization, for example, of the sterically most accessible 5-hydroxyl group (*18*). Much more experimental chemistry is required before an assessment of the chances of a synthetic selection of the RNA structure can be made, how-

**Table 1.** $T_m$ values and thermodynamic data of duplex formation. $T_m$ values refer to an overall oligomer concentration of 10 μM in 0.15 M NaCl, 0.01 M tris-HCl (pH 7.0) (values with asterisks indicate in 1.0 M NaCl); thermodynamic parameters were determined from plots of $T_m^{-1}$ versus ln c [for method see (*20*)]; experimental error for $\Delta H$ was estimated to be ±5%. Data for β-ribopyranosyl duplexes are from Bolli *et al.* (*5*) and from (*11*). RNA data determined for comparison (with T instead of U) were provided by T. Vivlemore (ETH). For the automated synthesis, isolation, and characterization of pentopyranosyl-(2'→4') oligonucleotides see (*5*) and (*7*).

| Octamer Duplex | Pentopyranosyl oligonucleotide system | $T_m$ (10 μM) °C in 0.15M NaCl | | ΔG 25° C kcal/mol | ΔH kcal/mol | TΔS 25° C kcal/mol |
|---|---|---|---|---|---|---|
| 4' 2' -AAAAAAAA TTTT TTTT- 2' 4' | β-ribo | 40 | 46.0* | -10.5 | -62.2 | -51.7 |
| | α-lyxo | 47.0 | 51.0* | -12.3 | -69.5 | -57.2 |
| | β-xylo | 35.4 | 47.3* | -8.2 | -39.3 | -31.1 |
| | α-arabino | 71.1 | 79.7* | -15.7 | -60.6 | -44.9 |
| | RNA | | 16.3* | -7.3* | -33.7* | -26.4* |
| -AAAATTTT TTTTAAAA- | β-ribo | 27 | | -7.3 | -48.1 | 40.8 |
| | α-lyxo | 38.2 | | -9.4 | -60.7 | -51.3 |
| | β-xylo | 16.3 | | -6.1 | -27.4 | -21.3 |
| | α-arabino | 61.2 | | -13.5 | -59.9 | -46.4 |
| | RNA | | 11.0* | -5.6* | -41.9* | -36.3* |
| -TTTTAAAA AAAATTTT- | β-ribo | 40 | | -9.8 | -59.9 | -50.1 |
| | α-lyxo | 47.0 | | -11.4 | -67.0 | -53.6 |
| | β-xylo | 40.3 | | -8.7 | -40.1 | -31.4 |
| | α-arabino | 69.4 | | -14.5 | -57.6 | -43.0 |
| | RNA | | 10.8* | -5.2* | -46.1* | -40.9* |
| -ATATATAT TATATATA- | β-ribo | 38 | | -9.2 | -58.7 | -49.5 |
| | α-lyxo | 38.3 | | -9.5 | -61.4 | -52.0 |
| | β-xylo | 28.6 | | -6.2 | -33.9 | -26.7 |
| | α-arabino | 60.0 | | --- | --- | --- |
| -TATATATA ATATATAT- | β-ribo | 40 | | -9.3 | -51.6 | -42.3 |
| | α-lyxo | 37.9 | | -9.4 | -62.9 | -53.5 |
| | β-xylo | 33.8 | | -7.6 | -28.7 | -21.1 |
| | α-arabino | 60.8 | | --- | --- | --- |




**Scheme 2.** $F_{moc}$ = 9-fluorenylmethoxycarbonyl; $A^{Bz}$ = 6-benzoylamino-purine-9-yl; T = thymine-1-yl; numbers before reagents denote mole equivalents, % denotes yield. Preparation of building blocks for oligonucleotide synthesis (*7*). (L)-α-Lyxopyranosyl series: **1a (1b)→2a (2b)**: 2.0 (2.5) triethylorthobenzoate, 0.9 (0.3) p-TsOH, DMF/$CH_3CN$, 30°C, 2 hours, 64% (74%); **2a (2b)→3a (3b)**: (a) 3.0 DMTrCl, 6 lutidine, $CH_2Cl_2$, 0°C, 8 hours; (b) 80 pct. $CH_3CO_2H$/THF/$CH_2Cl_2$ 1:2:2, 23°C, 55% (61%); **3a (3b)→4a (4b)**: 2.5 P((*i*-Pr)$_2$N)(OCH$_2$CH$_2$CN)Cl, 7.5 collidine, 0.5 N methylimidazole, $CH_2Cl_2$, 23°C, 10 min, 85% (80%). (D)-β-Xylopyranosyl series: **5a (5b)→6a (6b)**: 2.5 DMTrCl, pyridine, room temperature (RT), 12 hours, 38% (34%) after chromatographic separation from isomers; **6a (6b)→7a (7b)**: 1.5 chloroacetic acid anhydride, $CH_2Cl_2$/pyridine 4:1, 0°C, 1 hour, 45% (43%) after chromatography; **7a (7b)→8a (8b)**: $H_2$/10 pct. Pd(C), THF, 3.0 $K_2CO_3$, 96% (91%); **8a (8b)→9a (9b)**: 3.0 P((*i*-Pr)$_2$N)(OCH$_2$CH$_2$CN)Cl, 4.0 ethyldiisopropylamine, $CH_2Cl_2$, 23°C, 3 hours, 62% (81%). (D)-α-Arabinopyranosyl series: **10a→11a**: 3.0 2-methoxypropene, 0.03 TsOH, DMF, 0°C, 36 hours, 87%; **10b→11b**: 1.0 **10b** as Na salt, DMF, 1.2 HCl in dioxane, 1.7 2-methoxypropene, RT, 1.5 hours, 85%; **11a (11b)→12a (12b)**: (a) 1.0 $F_{moc}$Cl, pyridine/$CH_2Cl_2$ 3:1, 0°C→RT, 21 hours (1.5 hours), 87% (76%) of acetal derivative; (b) 80 pct. aqueous $CH_3CO_2H$, RT, 48 hours, 72% (0.026 M HCl in $CH_3OH$, 50°C, 2 hours, 74%). **12a (12b)→13a (13b)**: (a) 1.8 (1.2) BzCl, 4.8 (3.5) pyridine, $CH_2Cl_2$/THF 5:1 ($CH_2Cl_2$), 0°C→RT, 16 hours (3.5 hours), 69% (85%) of 3'-benzoyl derivative; (b) 2.5 P((*i*-Pr)$_2$N)(OCH$_2$CH$_2$CN)Cl, 0.5 imidazole, 4.0 *N*-methylmorpholine (0.5 collidine), THF, 1 hour, RT, 62% (60%). Solvents were dried by treatment with 4 Å molecular sieves. Experiments starting from arabinose have been carried out in the (D) series, which is enantiomeric to the series depicted in Schemes 1 and 2.

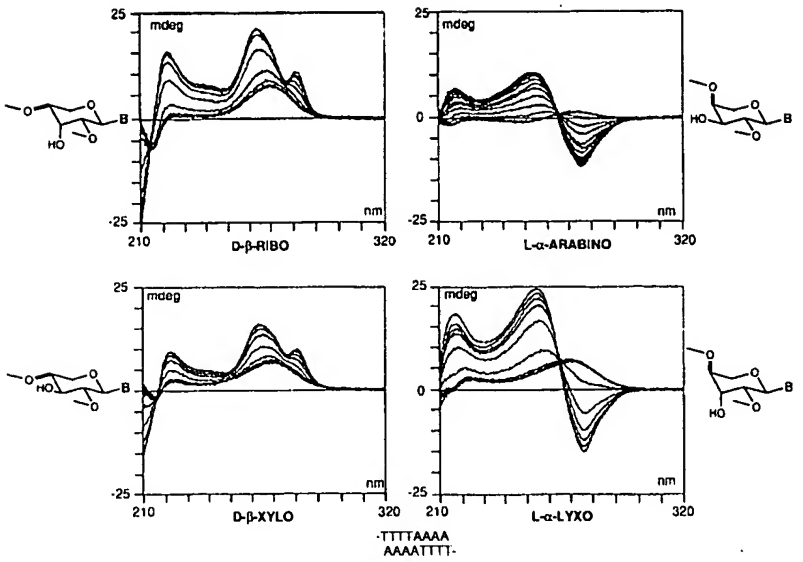



Fig. 2. Temperature-dependent CD spectra of the self-complementary sequences $T_4A_4$ in the four diastereomeric pentopyranosyl-(2′→4′) oligonucleotide systems [$c \approx 10$ μM, 0.15 M NaCl, 10 mM $NaH_2PO_4$, 0.1 mM $Na_2EDTA$ (pH 7.0)]. $T_m$ values derived from these CD curves: 41.1°C (β-ribo), 39.2°C (β-xylo), 45.4°C (α-lyxo), 66.1°C (α-arabino). CD spectrum of α-arabinopyranosyl duplex has been measured with a sample of the (D) series and is reproduced here as its mirror image.

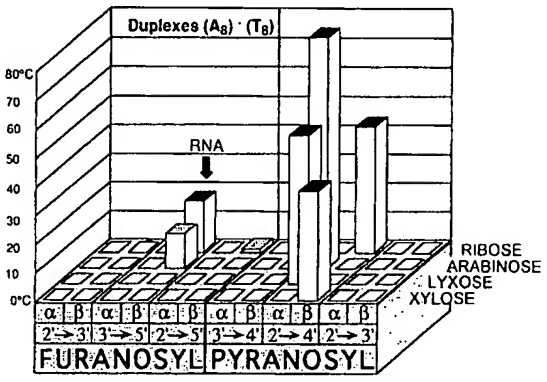



Fig. 3. Base-pairing–strength landscape of pentofuranosyl- and pentopyranosyl oligonucleotide systems showing the range of the constitutional and configurational diversity of (potentially natural) alternatives of the RNA structure and giving the $T_m$ values of $A_8 \cdot T_8$ duplexes of the systems investigated so far (5+5 μM, 1.0 M NaCl). Black top of columns: $T_m$ measured; shaded top of columns: $T_m$ estimated; empty squares: not investigated. The value for the β-arabinofuranosyl-(3′→5′) system is estimated from data provided by W. Pfleiderer (*21*), that of the ribofuranosyl-(2′→5′) system from data reported in (*2*). For the other $T_m$ values see Table 1.

ever. Finally, there is the possibility that RNA emerged within, or through the mediation of, an evolving biological system that later became extinct (*15*, *19*). In such a case, the determinant for RNA's selection could have been synthetic, functional, or both. Our conclusion regarding the role of pairing strength as a selection criterion for RNA remains valid independently of the actual determinant of RNA's selection.

The pairing-strength landscape shown in Fig. 3, in which natural RNA appears as a minor player, calls for charting other parts of the structural neighborhood of RNA with regard to its base-pairing potential. Within the pentopyranosyl domain, the members studied thus far are those that form preferentially under conditions of a conventional nucleosidation reaction and, therefore, may be of primary interest in an etiological context. However, the variants with inverted configuration at the anomeric center, or systems with phosphodiester junctions between positions other than 2′ and 4′, also deserve to be studied. An experimentally explored pairing-strength landscape of isomeric oligonucleotide systems would represent an exceptional source of reference data for a comprehensive analysis of the conformational and constellational factors that determine base pairing in nucleic acids. Our present data also may encourage attempts to map the complete pairing-strength landscape of RNA's structural neighborhood by theoretical methods.

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8. Details concerning preparation of building blocks and automated syntheses of oligomers in the three new pentopyranosyl-(2′→4′) oligonucleotide series will be published in *Helv. Chim. Acta* [F. Reck, H. Wippo, R. Kudick (L-α-lyxopyranosyl series); M. Beier and O. Jungmann (D- and L-α-arabinopyranosyl series); T. Wagner (D-β-xylopyranosyl series); R. Krishnamurthy and A. Eschenmoser, in preparation].
9. $T_m$ values of self-pairings (10 μM): $pl(A_8)$ 15°C and $pl(T_8)$ <5°C in 1.0 M NaCl; $pa(T_8)$ 17°C in 0.15 M NaCl and 28°C in 1.0 M NaCl; UV and CD of $pa(A_8)$ are essentially unchanged in the range 10° to 80°C (1.0 M NaCl).
10. $T_m$ values of cross-pairing (5 μM each strand, in 1.0 M NaCl): $pl(A_8)/pr(T_8)$ 31°C; $pl(T_8)/pr(A_8)$ 43°C; $pa(T_8)/pr(D_8)$ 55°C; $px(A_8)/pr(T_8)$ 46°C; $px(T_8)/pr(A_8)$ 45°C; $pl(A_8)/px(T_8)$ 27°C; $pl(T_8)/px(A_8)$ 42°C. Cross-pairing experiments involving the (D)-pa sequences have been carried out with (L)-pr sequences. No cross-pairing was observed between $A_8$ or $T_8$

strands of the (D)-pr-, (L)-pl-, and (D)-px series with complementary strands of the (D)-r series. For (L)-pr sequences, see (5). β-pr = ribo-, α-pl = lyxo-, β-px = xylo-, and α-pa = arabinopyranosyl; D = 2,6-diaminopurine.

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# Reduced Immunotoxicity and Preservation of Antibacterial Activity in a Releasable Side-Chain Carbapenem Antibiotic

**Hugh Rosen,* Richard Hajdu, Lynn Silver, Helmut Kropp, Karen Dorso, Joyce Kohler, Jon G. Sundelof, Joann Huber, Gail G. Hammond, Jesse J. Jackson, Charles J. Gill, Randall Thompson, Barbara A. Pelak, Jeffrey H. Epstein-Toney, George Lankas, Robert R. Wilkening, Kenneth J. Wildonger, Timothy A. Blizzard, Frank P. DiNinno, Ronald W. Ratcliffe, James V. Heck, John W. Kozarich, Milton L. Hammond**

A carbapenem antibiotic, L-786,392, was designed so that the side chain that provides high-affinity binding to the penicillin-binding proteins responsible for bacterial resistance was also the structural basis for ameliorating immunopathology. Expulsion of the side chain upon opening of the beta-lactam ring retained antibacterial activity while safely expelling the immunodominant epitope. L-786,392 was well tolerated in animal safety studies and had significant in vitro and in vivo activities against methicillin- and vancomycin-resistant Staphylococci and vancomycin-resistant Enterococci.

The increasing prevalence of resistance in Staphylococci and Enterococci to currently available antimicrobials has resulted in the significant diminution of therapeutic options available. Patients infected with multi–drug resistant organisms are once again succumbing to sepsis (*1*). One approach to counter this is to directly target the molecular mechanism of resistance in an existing class of antimicrobials, restoring their effectiveness. Methicillin resistance in Staphylococci is generally dependent on the production of a unique penicillin-binding protein (PBP). PBP2a, which like other high molecular weight PBPs, catalyzes the transpeptidation of peptidoglycan. PBP2a has a relatively low affinity to all the common β-lactam antibiotics and, in the presence of antibiotic, is able to take on the critical tasks of cell wall remodeling normally performed by the more antibiotic-susceptible PBPs (*2*).

Carbapenems acylate a broad spectrum of PBPs with high affinity. They are rapidly bactericidal, with potent activity against methicillin-sensitive staphylococci. These intrinsic properties, together with multiple PBP targets, result in low frequency of resistance selection. In addition, the carbapenem nucleus is resistant to most serine β-lactamases, whether of staphylococcal, enterococcal, or Gram-negative origin (*3*). We previously described a series of carbapenems with improved affinities for PBP2a, and concomitant activity against methicillin-resistant *Staphylococcus aureus* (MRSA) (*4*). Improved affinity for PBP2a was related to the presence of a large lipophilic substituent attached to the carbapenem nucleus. Unfortunately, carbapenems optimized for their PBP2a affinity provoked immune responses in rhesus monkeys (*5*), including high-frequency immune-mediated hemolytic anemia with Coombs positivity, a peripheral blood lymphocytosis, and diffuse lymphoid hyperplasia of spleen and lymph nodes at subhemolytic doses (15 mg/kg body weight/day of L-742,728). These nonanaphylactic autoimmune syndromes are occasionally observed with lower frequency and at higher doses in response to β-lactams in clinical use (*6*).

Sera from rhesus macaques exposed to the carbapenems L-742,728, L-741,462, and L-695,256 (*7*) all showed high titer, drug-specific antibodies that could be affinity purified on drug-sepharose columns and agglu-

Merck Research Laboratories, Rahway, NJ 07065, USA.

*To whom correspondence should be addressed. E-mail: hugh_rosen@merck.com

**Table 1.** Comparative in vitro antibacterial activity of L-786,392, imipenem, and vancomycin on Staphylococci and Enterococci.

| Organism (number of strains) | $MIC_{90}$ (μg/ml) | | |
|---|---|---|---|
| | L-786,392 | Vancomycin | Imipenem |
| Methicillin-susceptible *S. aureus* (15) | 0.06 | 2 | 0.03 |
| Methicillin-resistant *S. aureus* (42) | 4 | 2 | 256 |
| Methicillin-susceptible coagulase-negative *Staphylococcus* spp. (23) | 0.016 | 2 | 0.03 |
| Methicillin-resistant coagulase-negative *Staphylococcus* spp. (25) | 4 | 4 | 256 |
| Vancomycin-susceptible *E. faecalis* (17) | 1 | 2 | 1 |
| Vancomycin-resistant *E. faecalis* (15) | 4 | 1024 | 2 |
| Vancomycin-susceptible *E. faecium* (12) | 2 | 2 | 64 |
| Vancomycin-resistant *E. faecium* (31) | 8 | 2048 | >128 |
| *Enterococcus gallinarum* (14) | 1 | 8 | 16 |

# An invasive cleavage assay for direct quantitation of specific RNAs

Peggy S. Eis[1]*, Marilyn C. Olson[1], Tsetska Takova[1], Michelle L. Curtis[1], Sarah M. Olson[1], Tatiana I. Vener[1], Hon S. Ip[1], Kevin L. Vedvik[1], Christian T. Bartholomay[1], Hatim T. Allawi[1], Wu-Po Ma[1], Jeff G. Hall[1], Michelle D. Morin[1], Tom H. Rushmore[2], Victor I. Lyamichev[1], and Robert W. Kwiatkowski[1]

RNA quantitation is becoming increasingly important in basic, pharmaceutical, and clinical research. For example, quantitation of viral RNAs can predict disease progression and therapeutic efficacy[1]. Likewise, gene expression analysis of diseased versus normal, or untreated versus treated, tissue can identify relevant biological responses or assess the effects of pharmacological agents[2]. As the focus of the Human Genome Project moves toward gene expression analysis, the field will require a flexible RNA analysis technology that can quantitatively monitor multiple forms of alternatively transcribed and/or processed RNAs (refs 3,4). We have applied the principles of invasive cleavage[5] and engineered an improved 5′-nuclease to develop an isothermal, fluorescence resonance energy transfer (FRET)–based[6] signal amplification method for detecting RNA in both total RNA and cell lysate samples. This detection format, termed the RNA invasive cleavage assay, obviates the need for target amplification or additional enzymatic signal enhancement[7]. In this report, we describe the assay and present data demonstrating its capabilities for sensitive (<100 copies per reaction), specific (discrimination of 95% homologous sequences, 1 in ≥20,000), and quantitative (1.2-fold changes in RNA levels) detection of unamplified RNA in both single- and biplex-reaction formats.

A biplex format of the RNA invasive cleavage assay that enables simultaneous expression analysis of two genes within the same sample is shown in Figure 1. In the primary reaction (Fig. 1A), one-nucleotide overlap substrates[5] are generated by the hybridization of invasive deoxyoligonucleotides and probe deoxyoligonucleotides to their respective RNA targets. Each probe contains a specific, target-complementary region and a generic noncomplementary 5′-flap, which is released through 5′-nuclease cleavage. At the optimal reaction temperature, typically 60–65°C, the invasive oligonucleotides remain annealed to their RNA targets while the probes, which are present in large excess, undergo rapid dissociation and reassociation with the RNA targets[8,9]. In the presence of a 5′-nuclease, multiple probes are cleaved per invasive oligonucleotide:RNA target complex (probe turnover), resulting in target-specific accumulation of each generic-sequence 5′-flap. After completion of the primary reaction, the cleaved 5′-flaps then act as invasive oligonucleotides in a secondary reaction (Fig. 1B), in which they stably bind to the appropriate secondary-reaction template (SRT). Fluorescence signal is generated when multiple FRET deoxyoligonucleotides are cleaved per 5′-flap–SRT complex and is enhanced by the inclusion of arrestor oligonucleotides (Fig. 1B and below), which bind uncleaved probes. Specific detection of each RNA target is accomplished by using spectrally distinct fluorophores on the two FRET oligonucleotides. The detection format simplifies assay design and enables low-cost production because the probe's cleaved 5′-flap, the SRT, and the FRET oligonucleotide are not target-specific sequences. For example, we have used the same FRET oligonucleotide to measure >100 unique messenger RNAs (mRNAs) (data not shown).

The format of the RNA invasive cleavage assay differs from a similar assay for DNA detection[10] in several ways. First, it requires an alternate 5′-nuclease[8,11,12] that can cleave probes bound to RNA targets. The engineered variant used in this study, derived from *Thermus thermophilus* (*Tth*), contains three mutations (see Experimental Protocol) that yield enhanced activity on both RNA and DNA targets (data not shown). Second, unlike the 5′-nuclease used for DNA targets[8,10], the *Tth* 5′-nuclease allows sufficient signal amplification only when probe turnover occurs in both the primary and secondary reactions. Consequently, FRET signal detection requires two oligonucleotides (FRET and SRT) and a stably bound 5′-flap (Fig. 1B). Third, sequential operation of the two reactions is necessary to prevent the formation of "X-structures"[8]. These nonspecific substrates, which are stable uncleaved probe–SRT complexes, decrease signal generation by reducing the probe concentration in the primary reaction and preventing the formation of cleavable overlap substrates in the secondary reaction. The inclusion of 2′-*O*-methylated arrestor oligonucleotides, which are base-paired fully to each probe's target-specific region and partially to its 5′-flap region, sequesters uncleaved probes and prevents X-structure formation in the secondary reaction.

The two-step RNA invasive cleavage assay linearly amplifies signal in both a target- and time-dependent manner. In contrast, both reactions of the DNA invasive cleavage assay run concurrently and amplify signal as a linear function of target level, but as a quadratic function of time[10]. Any residual genomic DNA rarely affects assay results, because samples are not denatured. We have observed ~10-fold lower signal for nondenatured versus denatured genomic DNA, whereas total RNA samples yield nearly equivalent signal under both conditions (data not shown).

A key feature of the RNA invasive cleavage assay is its ability to discriminate highly homologous RNA sequences, such as those found in cytochrome P450 gene families[13]. To determine the assay's sensitivity and discrimination capabilities, we designed oligonucleotides for two 95% homologous mRNA targets[14], rat cytochrome P450 2B1 and 2B2 (Fig. 2A). Like the 5′-nuclease used for DNA detection[5], the *Tth* 5′-nuclease can discriminate single-base changes. To achieve this, we positioned the first complementary 5′-base of each probe at a nonconserved site in its mRNA target so that a mismatch would prevent formation of the overlap structure[5,8]. Additional nonconserved sites located in the probes, invasive oligonucleotides, or stacking oligonucleotides (see Fig. 2A legend) further enhanced discrimination. Designing assays with cleavage sites at particular exons or splice junctions enables detection of alternatively spliced mRNA variants (data not shown).

The 2B1 assay's sensitivity, determined with a 2B1 *in vitro* transcript, was 6,000 copies (0.01 attomol) per reaction (Fig. 2B). To examine cross-reactivity, we measured signal generated from excess 2B2 *in vitro* transcript in a 2B1 assay (Fig. 2B). Even at the highest level tested (5,000 attomol 2B2 transcript), no nonspecific signal was observed, indicating a minimum discrimination level of 1 in 500,000 (5,000/0.01 attomol). Analogous experiments performed with the 2B2 assay (Fig. 2C) indicated a discrimination level of 1 in 20,000 (1,000/0.05 attomol). Significantly, the 2B1 and 2B2 standard curves, measured in the absence or presence of ≥1,000 attomol

[1]*Third Wave Technologies, 502 South Rosa Road, Madison, WI 53719-1256.* [2]*Department of Drug Metabolism, Merck Research Laboratories, West Point, PA 19486.* **Corresponding author (peis@twt.com).*

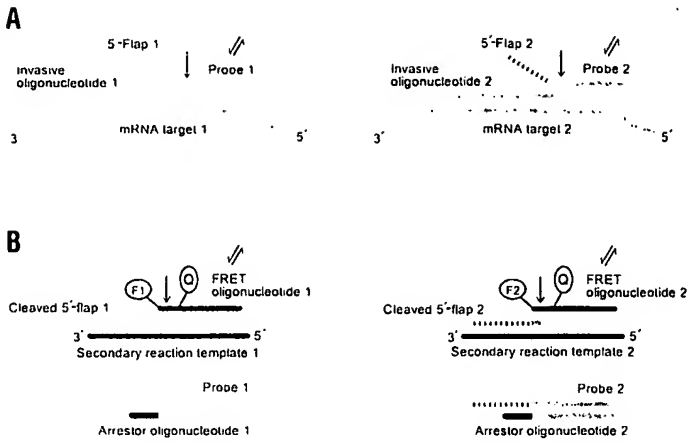


**Figure 1.** Schematic of a biplex-format RNA invasive cleavage assay. (A) Primary reaction: invasive oligonucleotides and probes specifically anneal to their respective RNA targets, forming different overlap structures. RNA targets and target-specific oligonucleotide sequences are shown in gray. Noncomplementary 5′-flaps are unshaded (5′-flap 1) or hatched (5′-flap 2). Vertical arrows indicate structure-specific 5′-nuclease cleavage, and double arrows indicate probe turnover. (B) Secondary reaction: cleaved 5′-flaps (generated in the primary reaction) and different-sequence FRET oligonucleotides form overlap structures by annealing to their respective SRTs. Each FRET oligonucleotide is labeled with a quencher molecule (Q) and a spectrally distinct fluorophore (F1 or F2). Vertical arrows indicate structure-specific 5′-nuclease cleavage, and double arrows indicate FRET oligonucleotide turnover. Cleavage between the fluorophore and quencher generates fluorescence signal. Except for each 5′-flap's 3′-nucleotide and the arrestor oligonucleotides' 5′-portions (target-specific regions, shown in gray), the secondary reaction sequences are generic (unshaded, hatched, or black).

nonspecific transcript, were nearly identical. Thus, the data (Figs. 2B,C) indicate that the assay can reliably detect low levels of a unique mRNA, even in samples containing high levels of closely related mRNAs. Cross-reactivity experiments performed with rat 3A1 and 3A2 assays and human 3A4, 3A5, and 3A7 (≥90% homologous sequences) indicated a minimal discrimination level of 1 in 200,000 (data not shown).

To validate the assay's quantitative performance, we tested four rat cytochrome P450s (2B1, 2B2, 3A1, 3A2) using total RNA prepared from four differentially treated samples (Ctrl, 3-MeC, Dex, PB; see Fig. 2D legend). A linear signal response was observed for all assays when measuring total RNA levels ranging from 0.1 to 250 ng (data not shown). The average coefficient of variation (c.v.) was 3% (representing 28 sample measurements, each in quadruplicate), with a range of 0.3–13%. Such precision is attributed to the assay's homogeneous, isothermal linear signal amplification. Figure 2D depicts the expression levels (mRNA copies/ng total RNA) for the assays with the four different samples, which range from 290 ± 40 for 2B1 (3-MeC) to 290,000 ± 11,000 for 3A1 (Dex). Statistical analysis (95% confidence intervals) of the fold changes (treated sample/untreated sample) in expression indicates that the assay can accurately discern changes as small as 1.2 fold (data not shown). To monitor large changes in mRNA levels, the dynamic range can be extended using real-time analysis. However, because the assay gen-

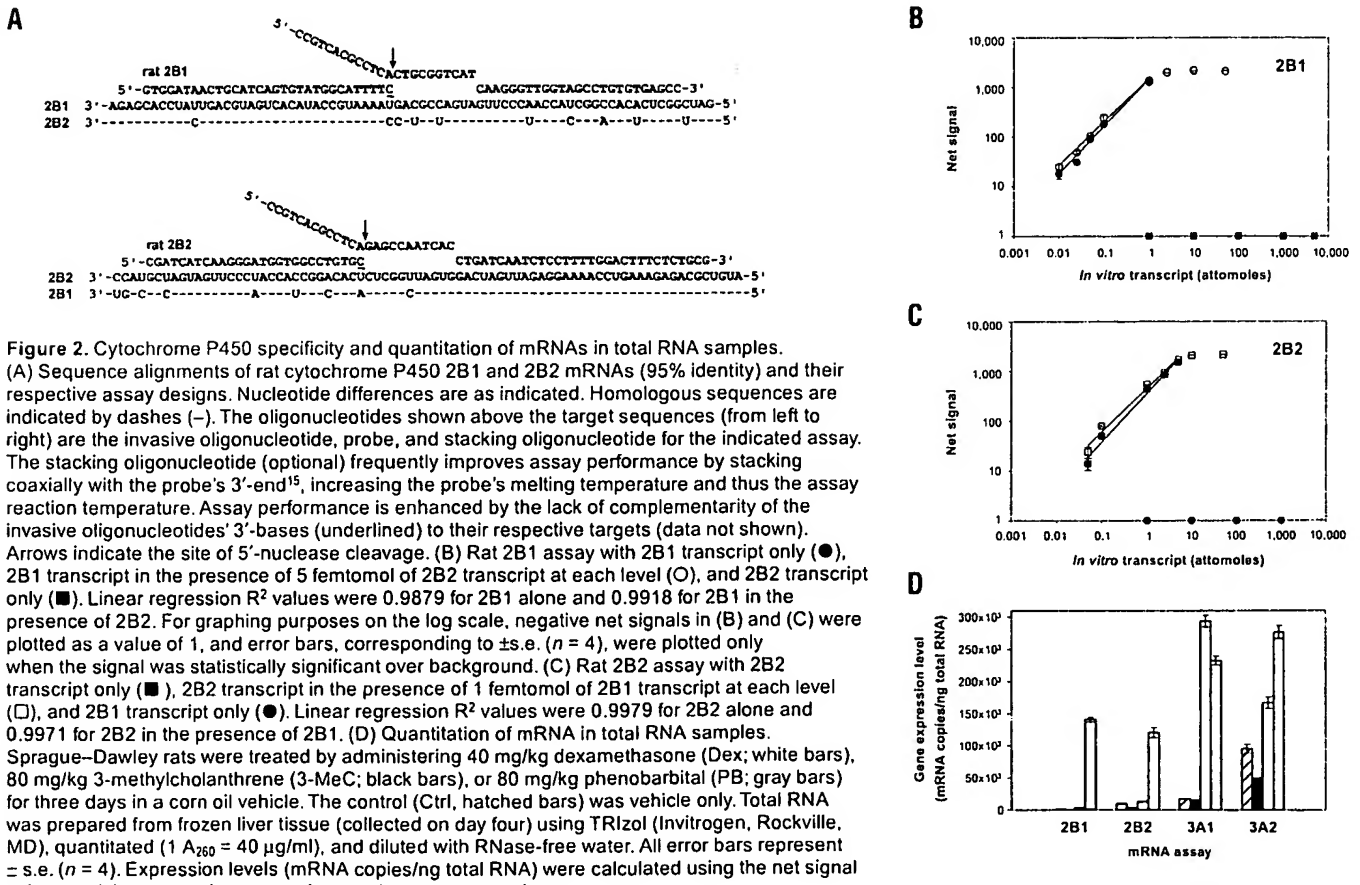


**Figure 2.** Cytochrome P450 specificity and quantitation of mRNAs in total RNA samples. (A) Sequence alignments of rat cytochrome P450 2B1 and 2B2 mRNAs (95% identity) and their respective assay designs. Nucleotide differences are as indicated. Homologous sequences are indicated by dashes (–). The oligonucleotides shown above the target sequences (from left to right) are the invasive oligonucleotide, probe, and stacking oligonucleotide for the indicated assay. The stacking oligonucleotide (optional) frequently improves assay performance by stacking coaxially with the probe's 3′-end[15], increasing the probe's melting temperature and thus the assay reaction temperature. Assay performance is enhanced by the lack of complementarity of the invasive oligonucleotides' 3′-bases (underlined) to their respective targets (data not shown). Arrows indicate the site of 5′-nuclease cleavage. (B) Rat 2B1 assay with 2B1 transcript only (●), 2B1 transcript in the presence of 5 femtomol of 2B2 transcript at each level (○), and 2B2 transcript only (■). Linear regression $R^2$ values were 0.9879 for 2B1 alone and 0.9918 for 2B1 in the presence of 2B2. For graphing purposes on the log scale, negative net signals in (B) and (C) were plotted as a value of 1, and error bars, corresponding to ±s.e. ($n$ = 4), were plotted only when the signal was statistically significant over background. (C) Rat 2B2 assay with 2B2 transcript only (■), 2B2 transcript in the presence of 1 femtomol of 2B1 transcript at each level (□), and 2B1 transcript only (●). Linear regression $R^2$ values were 0.9979 for 2B2 alone and 0.9971 for 2B2 in the presence of 2B1. (D) Quantitation of mRNA in total RNA samples. Sprague–Dawley rats were treated by administering 40 mg/kg dexamethasone (Dex; white bars), 80 mg/kg 3-methylcholanthrene (3-MeC; black bars), or 80 mg/kg phenobarbital (PB; gray bars) for three days in a corn oil vehicle. The control (Ctrl, hatched bars) was vehicle only. Total RNA was prepared from frozen liver tissue (collected on day four) using TRIzol (Invitrogen, Rockville, MD), quantitated (1 $A_{260}$ = 40 μg/ml), and diluted with RNase-free water. All error bars represent ± s.e. ($n$ = 4). Expression levels (mRNA copies/ng total RNA) were calculated using the net signal values and the appropriate transcript standard curve equation.

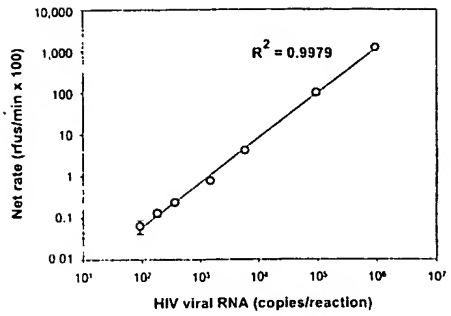


**Figure 3.** Real-time quantitation of HIV viral RNA. Viral particles were processed (see Experimental Protocol) and then diluted (from 940,000 to 90 copies/reaction) with the no-target control (100 ng tRNA/μl). The reported copies/reaction for the dilution series are derived from a blinded, independently validated (TriCore Reference Laboratories, Albuquerque, NM), HIV-spiked plasma sample ($1.36 \times 10^{11}$ copies/ml). Data were collected in quadruplicate on a real-time instrument and plotted as relative fluorescence units (rfus) versus time (min). Linear regression was performed over the full 120-min data collection period except for the two highest target levels (linear regressions performed at 50% maximal signal) to determine the rates of signal generation. Net rates were calculated by subtracting the average no-target rate from the average rate for each tested level in the dilution series. Detection of the lowest dilution sample (90 copies) was statistically significant by 99% confidence interval (no-target signal was 0.446 ± 0.058, 90-copy signal was 0.511 ± 0.003). Error bars represent ±s.e. ($n$ = 4).

erates signal linearly with time or target level, simply varying the amount of sample added per reaction and calculating the copies of mRNA per nanogram of total RNA enables accurate quantitation with a single endpoint measurement on low-cost instrumentation. Furthermore, in cases in which absolute quantitation is not necessary, the assay's linear signal amplification mechanism and reproducibility also eliminate the need for a standard curve and enable simple and accurate relative quantitation of any one gene. For example, we observed equivalent PB induction (160 fold) of 2B1 mRNA (data not shown) using either absolute quantitation, which required a standard curve, or comparison of net signal values (relative quantitation).

The analytical sensitivity of the cytochrome P450 mRNA assays (Fig. 2) ranges from 6,000 to 30,000 molecules per reaction, which is sufficient for analyzing changes in expression levels for many different genes. For additional sensitivity, we designed assays for accessible sites in the target RNA (ref. 16) that enable more efficient probe turnover and, thus, greater signal generation. Using the improved 5′-nuclease, this method allowed detection of as few as 90 copies of HIV-1 RNA (Fig. 3) prepared from viral particles (see Fig. 3 legend). A plot of average net rates (see Fig. 3 legend) for each dilution level (90–940,000 copy range) yielded a 4-log linear dose response over a 4-log range of target concentration.

Figure 4A compares the results of single versus biplex formats of the RNA invasive cleavage assay. Both formats yielded comparable dynamic ranges and limits of detection for both interleukin-8 (IL-8) RNA (0.01 attomol) and ubiquitin RNA (0.08 attomol). To simplify sample preparation, we developed a method to directly quantitate mRNA levels in cell lysates (Fig. 4B). An adherent cell line (MG-63) that upregulates expression of multiple cytokines[17] was used to validate the method. Ubiquitin was chosen as the internal control because its gene expression levels remained constant in untreated and treated cells in these experiments. Both IL-8 and ubiquitin signals increased linearly with increasing cell number, and were detected in the lowest cell number tested (~300 cells/reaction). Variation in the lysate samples was higher (c.v.'s 3–20%, average c.v. = 10%) than with the total RNA samples (c.v.'s 0.3–13%, average

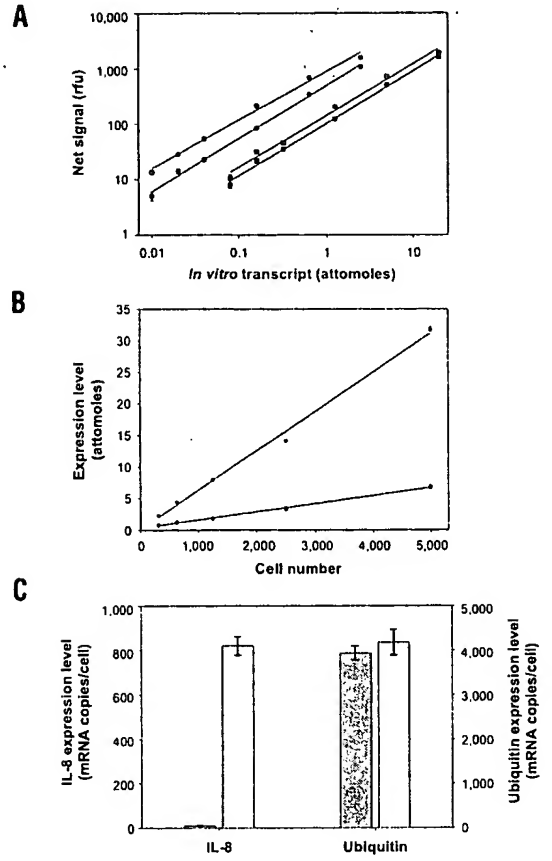


**Figure 4.** Biplex RNA invasive cleavage assay and detection of human IL-8 and ubiquitin mRNA in cell lysates. All error bars represent ±s.e. ($n$ = 4). (A) Single-reaction and biplex formats of IL-8 and ubiquitin assays on their respective transcripts: single IL-8 (○), biplex IL-8 (●), single ubiquitin (□), biplex ubiquitin (■). The $R^2$ values were 0.9927 (single IL-8), 0.9957 (biplex IL-8), 0.9906 (single ubiquitin) and 0.9957 (biplex ubiquitin). (B) Biplex detection of mRNAs in cell lysates prepared from serially diluted (40,000–2,500 cells per well, 96-well tissue culture) MG-63 cells (American Type Culture Collection, Manassas, VA). Before lysate preparation, cells were treated for 2 h with 10 ng/ml human TNF-α and 10 ng/ml human IL-1β (Calbiochem, La Jolla, CA). Medium was removed and the cells were washed 1× with 0.2 ml no-$MgCl_2$/no-$CaCl_2$ PBS (Invitrogen) without disrupting the monolayer. Cell lysate preparation: 40 μl lysis buffer (20 mM Tris pH8, 5mM $MgCl_2$, 0.5% NP-40, 200 mg/ml tRNA) was added per well, cells were lysed at room temperature for 3–5 min, 30 μl of each lysate sample were transferred to a 96-well microplate and cellular nucleases inactivated by heating the lysates for 15 min at 75–80°C. Lysate samples were immediately assayed (5 μl per reaction) or stored at –70°C before testing. Cell number corresponds to the number of cells in a 5-μl sample. Expression levels of IL-8 (●) and ubiquitin (■) were calculated using the net signal values and the appropriate transcript standard curve equation. Linear regression $R^2$ values were 0.9987 and 0.9959 for IL-8 and ubiquitin detection, respectively. (C) Detection of IL-8 and ubiquitin mRNA expression in untreated (dark gray shading) and treated (light gray shading) cells (5,000 cells/reaction).

c.v. = 3%). However, when the IL-8 signal from the lysate samples was normalized with the ubiquitin internal control signal, the precision was comparable (c.v.'s 1–6%, average c.v. = 3%). The biplex RNA invasive cleavage assay was also used to measure expression levels for IL-8 and ubiquitin in untreated and treated cells (Fig. 4C). As expected[18], IL-8 expression greatly increased in treated (820 mRNA copies/cell) versus untreated cells (11 mRNA copies/cell), indicating a 75-fold induction. However, ubiquitin expression remained relatively constant, showing only a 1.1-fold induction.

The RNA invasive cleavage assay is broadly applicable because of

its sensitivity, specificity, accuracy, and simple, low-cost format. Furthermore, it is particularly suited for detecting alternatively spliced or edited RNA variants[3,4,19] because even a single-base change at the overlap site affects 5′-nuclease cleavage, and assembly of the overlap structure requires only ~60 nucleotides of RNA sequence. The assay should be useful in any area requiring RNA quantitation, such as high-throughput screening in drug discovery research, monitoring of drug metabolism and safety in clinical trials, and clinical detection of viral RNA load.

## Experimental protocol

Generation of *Tth* 5′-nuclease. Recombinant polymerase-deficient *Tth* 5′-nuclease was cloned, expressed, and purified as described[11,20], except that it contained three additional mutations (H786A, G506K, Q509K). These mutations were based on previously reported literature, molecular modeling, and domain-swapping experiments[11]. Site-directed mutagenesis to create the mutations was performed with the Transformer Site-Directed Mutagenesis Kit (Clontech, Palo Alto, CA).

*In vitro* transcript generation. PCR products for rat 2B1, 2B2, and 3A1 RNA were generated from clones (provided by Merck Research Laboratories, West Point, PA) using the specified primers (see Supplementary Table 1, available in the Web Extras page of *Nature Biotechnology* Online). A short transcript comprising the binding region for the assay's oligonucleotides was generated for 3A2 from two synthetic oligonucleotides because the 3A2 clone was not available (Supplementary Table 1). Transcripts were generated using a T7-MEGAshortscript *in vitro* transcription kit (Ambion, Austin, TX), gel-purified on denaturing polyacrylamide gels, and quantitated by $A_{260}$ measurement. Transcripts were diluted with 20 ng/μl transfer RNA (tRNA) (Sigma, St. Louis, MO), which also served as the no-target control.

HIV RNA sample. Purified HIV viral particles (strain IIIB, $6.8 \times 10^{10}$ viral particles/ml ± 0.5 log unit; Advanced Biotechnologies, Inc., Columbia, MD) were diluted with HIV-negative plasma (Lampire Biological Laboratories, Pipersville, PA). Viral RNA was isolated from HIV-spiked plasma sample ($1.36 \times 10^{11}$ copies/ml) using a QIAamp Viral RNA Kit (QIAgen, Valencia, CA). Eluates ($2.7 \times 10^{10}$ copies/ml, assuming 100% recovery) were pooled and diluted with 100 ng/μl tRNA to generate the standards (see Fig. 4 legend). The 100 ng/μl tRNA solution was also used as the no-target control.

RNA invasive cleavage assays. All deoxyoligonucleotides were synthesized and HPLC-purified as described[10]. All oligonucleotides used in the assays are listed in Supplementary Table 1 (available in the Web Extras page of *Nature Biotechnology* Online). RNase-free water was used in all assay reagents. Primary reaction (10 μl volume) components were 10 mM MOPS (pH 7.5), 0.05% Tween-20, 0.05% Nonidet P-40 (NP-40), 4% polyethylene glycol (PEG), 100 mM KCl, 12.5 mM $MgSO_4$, 2 ng/μl *Tth* 5′-nuclease, 0.8 μM probe (1 μM for the HIV, IL-8, and ubiquitin assays), 0.5 μM invasive oligonucleotide, and 0.3 μM stacking oligonucleotide. Secondary reaction (15 μl volume) components were 10 mM MOPS (pH 7.5), 0.05% Tween-20, 0.05% NP-40, 20 mM $MgSO_4$, 0.67 μM FRET oligonucleotide, 0.1 μM SRT, 2.1 μM arrestor oligonucleotide (2.7 μM for HIV, IL-8, and ubiquitin assays); remaining primary reaction components were diluted 1.5×.

Primary reactions, except for HIV-1 (see below), were performed in a 96-well microplate (MJ Research, Waltham, MA), and to each well were added 5 μl primary reaction components (2× concentration), 5 μl standard or RNA sample, and 10 μl clear Chill-out 14 liquid wax (MJ Research). Microplates were incubated at 60°C for 90 min in a thermal cycler. To initiate the secondary reaction, 5 μl secondary reaction components (3× concentration) were added to each well and the microplate was further incubated at 60°C for 60 min. Microplates were directly read in a CytoFluor 4000 (PerSeptive Biosystems, Framingham, MA) using 485/20 nm excitation, 530/25 nm emission for FAM and 560/20 nm excitation, 620/40 nm emission for Redmond Red (Epoch Biosciences, Bothell, WA). The HIV reactions were performed as above, except in 0.2 ml tubes with primary reaction incubation (no Chill-out) in a heated-lid thermal cycler at 64°C for 120 min and secondary reaction incubation at 60°C for 120 min in a Rotor-Gene Real Time DNA Amplification System (Corbett Research, Sydney, Australia). Data were collected every 20 s for the first 10 min, every 1 min for the next 100 min, and every 5 min for the final 10 min (excitation 470 nm and emission 510 nm).

Data analysis. Data were exported into Microsoft Excel 97 software (Microsoft, Bellevue, WA). Net signal values were calculated by subtracting the average no-target signal from the average sample signal. The standard deviation (s.d.) and the percentage c.v. (%c.v. = s.d./average raw signal × 100) were calculated to determine variation in the replicates ($n = 4$). Results were significant if a *t*-test between the no-target replicates and the lowest level transcript replicates was $P \leq 0.05$. Dynamic ranges were determined by fitting the standard or RNA sample data with linear or power fit equations in SigmaPlot software (SPSS, Chicago, IL) to the range that yielded correlation coefficients ($R^2$) > 0.99.

*Note: Supplementary information can be found on the* Nature Biotechnology *website in Web Extras (http://biotech.nature.com/web_extras).*

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(71) Applicant *(for all designated States except US)*: EXIQON A/S [DK/DK]; Bygstubben 9, DK–2950 Vedbæk (DK).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: WENGEL, Jesper [DK/DK]; Rugmarken 48, DK–5260 Odense S. (DK). NIELSEN, Poul [DK/DK]; Elmevangen 6, DK–7200 Grindsted (DK).

(74) Agent: PLOUGMANN, VINGTOFT & PARTNERS; Sankt Annæ Plads 11, P.O. Box 3007, DK–1021 Copenhagen (DK).

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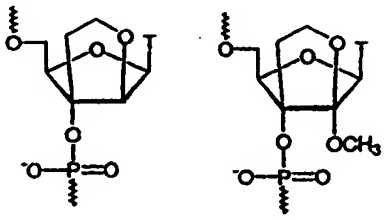


V X Y

zT: B = thymin-1-yl
zU: B = uracil-1-yl
zG: B = guanin-9-yl
zC: B = cytosin-1-yl
zA: B = adenin-9-yl
zMeC: B = 5-methylcytosin-1-yl

(57) Abstract

The present invention relates to novel bicyclic and tricyclic nucleoside and nucleotide analogues as well as to oligonucleotides comprising such elements. The nucleotide analogues, LNAs (Locked Nucleoside Analogues), are able to provide valuable improvements to oligonucleotides with respect to affinity and specificity towards complementary RNA and DNA oligomers. The novel type of LNA modified oligonucleotides, as well as the LNAs as such, are useful in a wide range of diagnostic applications as well as therapeutic applications. Among these can be mentionned antisense applications, PCR applications, strand displacement oligomers, as substrates for nucleic acid polymerases, as nucleotide based drugs, etc. The present invention also relates to such applications.

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# OLIGONUCLEOTIDE ANALOGUES

## FIELD OF THE INVENTION

The present invention relates to the field of bi- and tricyclic nucleoside analogues and to the synthesis of such nucleoside analogues which are useful in the formation of synthetic oligonucleotides capable of forming nucleobase specific duplexes and triplexes with single stranded and double stranded nucleic acids. These complexes exhibit higher thermostability than the corresponding complexes formed with normal nucleic acids. The invention also relates to the field of bi- and tricyclic nucleoside analogues and the synthesis of such nucleosides which may be used as therapeutic drugs and which may be incorporated in oligonucleotides by template dependent nucleic acid polymerases.

## BACKGROUND OF THE INVENTION

Synthetic oligonucleotides are widely used compounds in disparate fields such as molecular biology and DNA-based diagnostics and therapeutics.

### Therapeutics

In therapeutics, *e.g.*, oligonucleotides have been used successfully to block translation in vivo of specific mRNAs thereby preventing the synthesis of proteins which are undesired or harmful to the cell/organism. This concept of oligonucleotide mediated blocking of translation is known as the "antisense" approach. Mechanistically, the hybridising oligonucleotide is thought to elicit its effect by either creating a physical block to the translation process or by recruiting cellular enzymes that specifically degrades the mRNA part of the duplex (RNAseH).

More recently, oligoribonucleotides and oligodeoxyribonucleotides and analogues thereof which combine RNAse catalytic activity with the ability to sequence specifically interact with a complementary RNA target (ribozymes) have attracted

much interest as antisense probes. Thus far ribozymes have been reported to be effective in cell cultures against both viral targets and oncogenes.

To completely prevent the synthesis of a given protein by the antisense approach it is necessary to block/destroy all mRNAs that encode that particular protein and in many cases the number of these mRNA are fairly large. Typically, the mRNAs that encode a particular protein are transcribed from a single or a few genes. Hence, by targeting the gene ("antigene" approach) rather than its mRNA products it should be possible to either block production of its cognate protein more efficiently or to achieve a significant reduction in the amount of oligonucleotides necessary to elicit the desired effect. To block transcription, the oligonucleotide must be able to hybridise sequence specifically to double stranded DNA. In 1953 Watson and Crick showed that deoxyribo nucleic acid (DNA) is composed of two strands (Nature, 1953, 171, 737) which are held together in a helical configuration by hydrogen bonds formed between opposing complementary nucleobases in the two strands. The four nucleobases, commonly found in DNA are guanine (G), adenine (A), thymine (T) and cytosine (C) of which the G nucleobase pairs with C, and the A nucleobase pairs with T. In RNA the nucleobase thymine is replaced by the nucleobase uracil (U) which similarly to the T nucleobase pairs with A. The chemical groups in the nucleobases that participate in standard duplex formation constitute the Watson-Crick face. In 1959, Hoogsteen showed that the purine nucleobases (G and A) in addition to their Watson-Crick face have a Hoogsteen face that can be recognised from the outside of a duplex, and used to bind pyrimidine oligonucleotides via hydrogen bonding, thereby forming a triple helix structure. Although making the "antigene" approach conceptually feasible the practical usefulness of triple helix forming oligomers is currently limited by several factors including the requirement for homopurine sequence motifs in the target gene and a need for unphysiologically high ionic strength and low pH to stabilise the complex.

The use of oligonucleotides known as aptamers are also being actively investigated. This promising new class of therapeutic oligonucleotides are selected *in vitro* to specifically bind to a given target with high affinity, such as for example ligand receptors. Their binding characteristics are likely a reflection of the ability of oligonucleotides to form three dimensional structures held together by intramolecular nucleobase pairing.

Likewise, nucleosides and nucleoside analogues have proven effective in chemotherapy of numerous viral infections and cancers.

Also, various types of double-stranded RNAs have been shown to effectively inhibit the growth of several types of cancers.

**Diagnostics**

In molecular biology, oligonucleotides are routinely used for a variety of purposes such as for example (i) as hybridisation probes in the capture, identification and quantification of target nucleic acids (ii) as affinity probes in the purification of target nucleic acids (iii) as primers in sequencing reactions and target amplification processes such as the polymerase chain reaction (PCR) (iv) to clone and mutate nucleic acids and (vi) as building blocks in the assembly of macromolecular structures.

Diagnostics utilises many of the oligonucleotide based techniques mentioned above in particular those that lend themselves to easy automation and facilitate reproducible results with high sensitivity. The objective in this field is to use oligonucleotide based techniques as a means to, for example (i) tests humans, animals and food for the presence of pathogenic micro-organisms (ii) to test for genetic predisposition to a disease (iii) to identify inherited and acquired genetic disorders, (iv) to link biological deposits to suspects in crime trials and (v) to validate the presence of micro-organisms involved in the production of foods and beverages.

**General considerations**

To be useful in the extensive range of different applications outlined above, oligonucleotides have to satisfy a large number of different requirements. In antisense therapeutics, for instance, a useful oligonucleotide must be able to penetrate the cell membrane, have good resistance to extra- and intracellular nucleases and preferably have the ability to recruit endogenous enzymes like RNAseH. In DNA-based diagnostics and molecular biology other properties are important such as, *e.g.*, the ability of oligonucleotides to act as efficient substrates for a wide range of different enzymes evolved to act on natural nucleic acids, such as *e.g.* polymerases, kinases,

ligases and phosphatases. The fundamental property of oligonucleotides, however, which underlies all uses is their ability to recognise and hybridise sequence specifically to complementary single stranded nucleic acids employing either Watson-Crick hydrogen bonding (A-T and G-C) or other hydrogen bonding schemes such as the Hoogsteen mode. The are two important terms affinity and specificity are commonly used to characterise the hybridisation properties of a given oligonucleotide. Affinity is a measure of the binding strength of the oligonucleotide to its complementary target sequence (expressed as the thermostability ($T_m$) of the duplex). Each nucleobase pair in the duplex adds to the thermostability and thus affinity increases with increasing size (No. of nucleobases) of the oligonucleotide. Specificity is a measure of the ability of the oligonucleotide to discriminate between a fully complementary and a mismatched target sequence. In other words, specificity is a measure of the loss of affinity associated with mismatched nucleobase pairs in the target. At constant oligonucleotide size the specificity increases with increasing number of mismatches between the oligonucleotide and its targets (*i.e.* the percentage of mismatches increases). Conversely, specificity decreases when the size of the oligonucleotide is increased at a constant number of mismatches (*i.e.* the percentage of mismatches decreases). Stated another way, an increase in the affinity of an oligonucleotide occurs at the expense of specificity and vice-versa.

This property of oligonucleotides creates a number of problems for their practical use. In lengthy diagnostic procedures, for instance, the oligonucleotide needs to have both high affinity to secure adequate sensitivity of the test and high specificity to avoid false positive results. Likewise, an oligonucleotide used as antisense probes needs to have both high affinity for its target mRNA to efficiently impair its translation and high specificity to avoid the unintentional blocking of the expression of other proteins. With enzymatic reactions, like, *e.g.*, PCR amplification, the affinity of the oligonucleotide primer must be high enough for the primer/target duplex to be stable in the temperature range where the enzymes exhibits activity, and specificity needs to be high enough to ensure that only the correct target sequence is amplified.

Given the shortcomings of natural oligonucleotides, new approaches for enhancing specificity and affinity would be highly useful for DNA-based therapeutics, diagnostics and for molecular biology techniques in general.

**Conformationally restricted nucleosides**

It is known that oligonucleotides undergo a conformational transition in the course of hybridising to a target sequence, from the relatively random coil structure of the single stranded state to the ordered structure of the duplex state.

A number of conformationally restricted oligonucleotides including bicyclic and tricyclic nucleoside analogues (Figure 1A and 1B in which B=nucleobase) have been synthesised, incorporated into oligonucleotide and oligonucleotide analogues and tested for their hybridisation and other properties.

Bicyclo[3.3.0] nucleosides (bcDNA) with an additional C-3´,C-5´-ethano-bridge (**A** and **B**) have been synthesised with all five nucleobases (G, A, T, C and U) whereas (**C**) has been synthesised only with T and A nucleobases (M. Tarköy, M. Bolli, B. Schweizer and C. Leumann, *Helv. Chim. Acta*, 1993, **76**, 481; Tarköy and C. Leumann, *Angew. Chem., Int. Ed. Engl.*, 1993, **32**, 1432; M. Egli, P. Lubini, M. Dobler and C. Leumann, *J. Am. Chem. Soc.*, 1993, **115**, 5855; M. Tarköy, M. Bolli and C. Leumann, *Helv. Chim. Acta*, 1994, **77**, 716; M. Bolli and C. Leumann, *Angew. Chem., Int. Ed. Engl.*, 1995, **34**, 694; M. Bolli, P. Lubini and C. Leumann, *Helv. Chim. Acta*, 1995, **78**, 2077; J. C. Litten, C. Epple and C. Leumann, *Bioorg. Med. Chem. Lett.*, 1995, **5**, 1231; J. C. Litten and C. Leumann, *Helv. Chim. Acta*, 1996, **79**, 1129; M. Bolli, J. C. Litten, R. Schültz and C. Leumann, *Chem. Biol.*, 1996, **3**, 197; M. Bolli, H. U. Trafelet and C. Leumann, *Nucleic Acids Res.*, 1996, **24**, 4660). DNA oligonucleotides containing a few, or being entirely composed, of these analogues are in most cases able to form Watson-Crick bonded duplexes with complementary DNA and RNA oligonucleotides. The thermostability of the resulting duplexes, however, is either distinctly lower (**C**), moderately lower (**A**) or comparable to (**B**) the stability of the natural DNA and RNA counterparts. All bcDNA oligomers exhibited a pronounced increase in sensitivity to the ionic strength of the hybridisation media compared to the natural counterparts. The α-bicyclo-DNA (**B**) is more stable towards the 3´-exonuclease snake venom phosphordiesterase than the β-bicyclo-DNA (**A**) which is only moderately more stable than unmodified oligonucleotides.

Bicarbocyclo[3.1.0]nucleosides with an additional C-1´,C-6´- or C-6´,C-4´-methano-bridge on a cyclopentane ring (**D** and **E**, respectively) have been synthesised with all five nucleobases (T, A, G, C and U). Only the T-analogues, however, have been incorporated into oligomers. Incorporation of one or ten monomers **D** in a mixed poly-pyrimidine DNA oligonucleotide resulted in a substantial decrease in the affinity towards both DNA and RNA oligonucleotides compared to the unmodified reference oligonucleotide. The decrease was more pronounced with ssDNA than with ssRNA. Incorporation of one monomer **E** in two different poly-pyrimidine DNA oligonucleotides induced modest increases in $T_m$'s of 0.8 °C and 2.1 °C for duplexes towards ssRNA compared with unmodified reference duplexes. When ten T-analogues were incorporated into a 15mer oligonucleotide containing exclusively phosphorothioate internucleoside linkages, the $T_m$ against the complementary RNA oligonucleotide was increased approximately 1.3 °C per modification compared to the same unmodified phosphorothioate sequence. Contrary to the control sequence the oligonucleotide containing the bicyclic nucleoside **E** failed to mediate RNAseH cleavage. The hybridisation properties of oligonucleotides containing the G, A, C and U-analogues of **E** have not been reported. Also, the chemistry of this analogue does not lend itself to further intensive investigations on completely modified oligonucleotides (K.-H. Altmann, R. Kesselring, E. Francotte and G. Rihs, *Tetrahedron Lett.*, 1994, **35**, 2331; K.-H. Altmann, R. Imwinkelried, R. Kesselring and G. Rihs, *Tetrahedron Lett.*, 1994, **35**, 7625; V. E. Marquez, M. A. Siddiqui, A. Ezzitouni, P. Russ, J. Wang, R. W. Wagner and M. D. Matteucci, *J. Med. Chem.*, 1996, **39**, 3739; A. Ezzitouni and V. E. Marquez, *J. Chem. Soc., Perkin Trans. 1*, 1997, 1073).

A bicyclo[3.3.0] nucleoside containing an additional C-2´,C-3´-dioxalane ring has been synthesised as a dimer with an unmodified nucleoside where the additional ring is part of the internucleoside linkage replacing a natural phosphordiester linkage (**F**). This analogue was only synthesised as either thymine-thymine or thymine-5-methylcytosine blocks. A 15-mer polypyrimidine sequence containing seven of these dimeric blocks and having alternating phosphordiester- and riboacetal-linkages, exhibited a substantially decreased $T_m$ against complementary ssRNA compared to a control sequence with exclusively natural phosphordiester internucleoside linkages (R. J. Jones, S. Swaminathan, J. F. Millagan, S. Wadwani, B. S. Froehler and M. Matteucci, *J. Am. Chem. Soc.*, 1993, **115**, 9816).

The two dimers (**G** and **H**) with additional C-2´,C-3´-dioxane rings forming bicyclic[4.3.0]-systems in acetal-type internucleoside linkages have been synthesised as T-T dimers and incorporated once in the middle of 12mer polypyrimidine oligonucleotides. Oligonucleotides containing either **G** or **H** both formed significantly less stable duplexes with complementary ssRNA and ssDNA compared with the unmodified control oligonucleotide (J. Wang and M. D. Matteucci, *Bioorg. Med. Chem. Lett.*, 1997, **7**, 229).

Dimers containing a bicyclo[3.1.0]nucleoside with a C-2´,C-3´-methano bridge as part of amide- and sulfonamide-type (**I** and **J**) internucleoside linkages have been synthesised and incorporated into oligonucleotides. Oligonucleotides containing one ore more of these analogues showed a significant reduction in $T_m$ compared to unmodified natural oligonucleotide references (C. G. Yannopoulus, W. Q. Zhou, P. Nower, D. Peoch, Y. S. Sanghvi and G. Just, *Synlett*, 1997, 378).

A trimer with formacetal internucleoside linkages and a bicyclo[3.3.0] glucose-derived nucleoside analogue in the middle (**K**) has been synthesised and connected to the 3´-end of an oligonucleotide. The $T_m$ against complementary ssRNA was decreased by 4 °C, compared to a control sequence, and by 1.5 °C compared to a sequence containing two 2´,5´-formacetal linkages in the 3´-end (C. G. Yannopoulus, W. Q. Zhou, P. Nower, D. Peoch, Y. S. Sanghvi and G. Just, *Synlett*, 1997, 378).

Very recently oligomers composed of tricyclic nucleoside-analogues (**L**) have been reported to show increased duplex stability compared to natural DNA (R. Steffens and C. Leumann (Poster SB-B4), *Chimia*, 1997, **51**, 436).

Three bicyclic ([4.3.0] and [3.3.0]) nucleosides with an additional C-2´,C-3´-connected six- (**M** and **N**) or five-membered ring (**O**) have been synthesised as the T-analogues. The bicyclic nucleosides **M** and **N** have been incorporated once and twice into 14-mer oligo-T sequences. The $T_m$'s against complementary ssRNA and ssDNA were decreased by 6-10 °C per modification compared to unmodified control sequences. Fully modified oligonucleotides of analogue **O** exhibited an increased $T_m$ of approximately 1.0 °C per modification against the complementary RNA oligonucleotide

compared to the control DNA oligonucleotide. Also, the fully modified sequence was substantially more stable towards snake-venom phosphordiesterase hydrolysis than the unmodified control sequence. Partly modified oligonucleotides in which up to four analogues of **O** were incorporated, however, were less thermostable than the corresponding unmodified oligonucleotides. All oligonucleotides containing analogue **O** (both fully and partly modified) showed a substantial decrease in thermostability against complementary DNA oligonucleotides compared to the unmodified oligonucleotides (P. Nielsen, H. M. Pfundheller, J. Wengel, *Chem. Commun.*, 1997, 826; P. Nielsen, H. M. Pfundheller, J. Wengel, XII International Roundtable: Nucleosides, Nucleotides and Their Biological Applications; La Jolla, California, September 15-19, 1996; Poster PPI 43).

An attempt to make the bicyclic uridine nucleoside analogue **Q** planned to contain an additional O-2',C-4'-five-membered ring, starting from 4'-C-hydroxymethyl nucleoside **P**, failed (K. D. Nielsen, *Specialerapport (Odense University, Denmark)*, 1995).

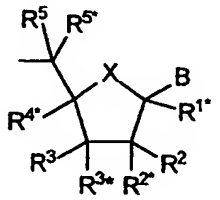
Until now the pursuit of conformationally restricted nucleosides useful in the formation of synthetic oligonucleotides with significantly improved hybridisation characteristics has met with little success. In the majority of cases, oligonucleotides containing these analogues form less stable duplexes with complementary nucleic acids compared to the unmodified oligonucleotides. In other cases, where moderate improvement in duplex stability is observed, this relates only to either a DNA or an RNA target, or it relates to fully but not partly modified oligonucleotides or vice versa. An appraisal of most of the reported analogues are further complicated by the lack of data on analogues with G, A and C nucleobases and lack of data indicating the specificity and mode of hybridisation. In many cases, synthesis of the reported monomer analogues is very complex while in other cases the synthesis of fully modified oligonucleotides is incompatible with the widely used phosphoramidite chemistry standard.

## SUMMARY OF THE INVENTION

In view of the shortcomings of the previously known nucleoside analogues, the present inventors have now provided novel nucleoside analogues (LNAs) and oligonucleotides have included LNA nucleoside analogues therein. The novel LNA

nucleoside analogues have been provided with all commonly used nucleobases thereby providing a full set of nucleoside analogues for incorporation in oligonucleotides. As will be apparent from the following, the LNA nucleoside analogues and the LNA modified oligonucleotide provides a wide range of improvements for oligonucleotides used in the fields of diagnostics and therapy. Furthermore, the LNA nucleoside analogues and the LNA modified oligonucleotide also provides completely new perspectives in nucleoside and oligonucleotide based diagnostics and therapy.

Thus, the present invention relates to oligomers comprising at least one nucleoside analogue (hereinafter termed "LNA") of the general formula I



I

wherein X is selected from -O-, -S-, -N($R^{N*}$)-, -C($R^6R^{6*}$)-, -O-C($R^7R^{7*}$)-, -C($R^6R^{6*}$)-O-, -S-C($R^7R^{7*}$)-, -C($R^6R^{6*}$)-S-, -N($R^{N*}$)-C($R^7R^{7*}$)-, -C($R^6R^{6*}$)-N($R^{N*}$)-, and -C($R^6R^{6*}$)-C($R^7R^{7*}$)-;

B is selected from hydrogen, hydroxy, optionally substituted $C_{1-4}$-alkoxy, optionally substituted $C_{1-4}$-alkyl, optionally substituted $C_{1-4}$-acyloxy, nucleobases, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands;

P designates the radical position for an internucleoside linkage to a succeeding monomer, or a 5'-terminal group, such internucleoside linkage or 5'-terminal group optionally including the substituent $R^5$;

one of the substituents $R^2$, $R^{2*}$, $R^3$, and $R^{3*}$ is a group P* which designates an internucleoside linkage to a preceding monomer, or a 3'-terminal group;

one or two pairs of non-geminal substituents selected from the present substituents of $R^{1*}$, $R^{4*}$, $R^5$, $R^{5*}$, $R^6$, $R^{6*}$, $R^7$, $R^{7*}$, $R^{N*}$, and the ones of $R^2$, $R^{2*}$, $R^3$, and $R^{3*}$ not

designating P* each designates a biradical consisting of 1-8 groups/atoms selected from -C($R^aR^b$)-, -C($R^a$)=C($R^a$)-, -C($R^a$)=N-, -O-, -Si($R^a$)$_2$-, -S-, -$SO_2$-, -N($R^a$)-, and >C=Z, wherein Z is selected from -O-, -S-, and -N($R^a$)-, and $R^a$ and $R^b$ each is independently selected from hydrogen, optionally substituted $C_{1-12}$-alkyl, optionally substituted $C_{2-12}$-alkenyl, optionally substituted $C_{2-12}$-alkynyl, hydroxy, $C_{1-12}$-alkoxy, $C_{2-12}$-alkenyloxy, carboxy, $C_{1-12}$-alkoxycarbonyl, $C_{1-12}$-alkylcarbonyl, formyl, aryl, aryloxy-carbonyl, aryloxy, arylcarbonyl, heteroaryl, heteroaryloxy-carbonyl, heteroaryloxy, heteroarylcarbonyl, amino, mono- and di($C_{1-6}$-alkyl)amino, carbamoyl, mono- and di($C_{1-6}$-alkyl)-amino-carbonyl, amino-$C_{1-6}$-alkyl-aminocarbonyl, mono- and di($C_{1-6}$-alkyl)amino-$C_{1-6}$-alkyl-aminocarbonyl, $C_{1-6}$-alkyl-carbonylamino, carbamido, $C_{1-6}$-alkanoyloxy, sulphono, $C_{1-6}$-alkylsulphonyloxy, nitro, azido, sulphanyl, $C_{1-6}$-alkylthio, halogen, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, where aryl and heteroaryl may be optionally substituted, and where two geminal substituents $R^a$ and $R^b$ together may designate optionally substituted methylene (=$CH_2$), and wherein two non-geminal or geminal substitutents selected from $R^a$, $R^b$, and any of the substituents $R^{1*}$, $R^2$, $R^{2*}$, $R^3$, $R^{3*}$, $R^{4*}$, $R^5$, $R^{5*}$, $R^6$ and $R^{6*}$, $R^7$, and $R^{7*}$ which are present and not involved in P, P* or the biradical(s) together may form an associated biradical selected from biradicals of the same kind as defined before; said pair(s) of non-geminal substituents thereby forming a mono- or bicyclic entity together with (i) the atoms to which said non-geminal substituents are bound and (ii) any intervening atoms; and

each of the substituents $R^{1*}$, $R^2$, $R^{2*}$, $R^3$, $R^{4*}$, $R^5$, $R^{5*}$, $R^6$ and $R^{6*}$, $R^7$, and $R^{7*}$ which are present and not involved in P, P* or the biradical(s), is independently selected from hydrogen, optionally substituted $C_{1-12}$-alkyl, optionally substituted $C_{2-12}$-alkenyl, optionally substituted $C_{2-12}$-alkynyl, hydroxy, $C_{1-12}$-alkoxy, $C_{2-12}$-alkenyloxy, carboxy, $C_{1-12}$-alkoxycarbonyl, $C_{1-12}$-alkylcarbonyl, formyl, aryl, aryloxy-carbonyl, aryloxy, arylcarbonyl, heteroaryl, heteroaryloxy-carbonyl, heteroaryloxy, heteroarylcarbonyl, amino, mono- and di($C_{1-6}$-alkyl)amino, carbamoyl, mono- and di($C_{1-6}$-alkyl)-amino-carbonyl, amino-$C_{1-6}$-alkyl-aminocarbonyl, mono- and di($C_{1-6}$-alkyl)amino-$C_{1-6}$-alkyl-aminocarbonyl, $C_{1-6}$-alkyl-carbonylamino, carbamido, $C_{1-6}$-alkanoyloxy, sulphono, $C_{1-6}$-alkylsulphonyloxy, nitro, azido, sulphanyl, $C_{1-6}$-alkylthio, halogen, DNA intercalators,

photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, where aryl and heteroaryl may be optionally substituted, and where two geminal substituents together may designate oxo, thioxo, imino, or optionally substituted methylene, or together may form a spiro biradical consisting of a 1-5 carbon atom(s) alkylene chain which is optionally interrupted and/or terminated by one or more heteroatoms/groups selected from -O-, -S-, and -($NR^N$)- where $R^N$ is selected from hydrogen and $C_{1-4}$-alkyl, and where two adjacent (non-geminal) substituents may designate an additional bond resulting in a double bond; and $R^{N*}$, when present and not involved in a biradical, is selected from hydrogen and $C_{1-4}$-alkyl;

and basic salts and acid addition salts thereof;

with the proviso that,

(i) $R^2$ and $R^3$ do not together designate a biradical selected from -O-$CH_2$-$CH_2$- and -O-$CH_2$-$CH_2$-$CH_2$- when LNA is a bicyclic nucleoside analogue;

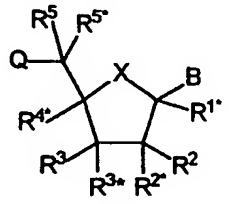
(ii) $R^3$ and $R^5$ do not together designate a biradical selected from -$CH_2$-$CH_2$-, -O-$CH_2$-, when LNA is a bicyclic nucleoside analogue;

(iii) $R^3$, $R^5$, and $R^{5*}$ do not together designate a triradical -$CH_2$-CH(-)-$CH_2$- when LNA is a tricyclic nucleoside analogue;

(iv) $R^{1*}$ and $R^{6*}$ do not together designate a biradical -$CH_2$- when LNA is a bicyclic nucleoside analogue; and

(v) $R^{4*}$ and $R^{6*}$ do not together designate a biradical -$CH_2$- when LNA is a bicyclic nucleoside analogue.

The present invention furthermore relates to nucleoside analogues (hereinafter LNAs) of the general formula II




II

wherein the substituent B is selected from nucleobases, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands;

X is selected from -O-, -S-, -N($R^{N*}$)-, and -C($R^6R^{6*}$)-;

one of the substituents $R^2$, $R^{2*}$, $R^3$, and $R^{3*}$ is a group Q*;

each of Q and Q* is independently selected from hydrogen, azido, halogen, cyano, nitro, hydroxy, Prot-O-, Act-O-, mercapto, Prot-S-, Act-S-, $C_{1-6}$-alkylthio, amino, Prot-N($R^H$)-, Act-N($R^H$)-, mono- or di($C_{1-6}$-alkyl)amino, optionally substituted $C_{1-6}$-alkoxy, optionally substituted $C_{1-6}$-alkyl, optionally substituted $C_{2-6}$-alkenyl, optionally substituted $C_{2-6}$-alkenyloxy, optionally substituted $C_{2-6}$-alkynyl, optionally substituted $C_{2-6}$-alkynyloxy, monophosphate, diphosphate, triphosphate, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, ligands, carboxy, sulphono, hydroxymethyl, Prot-O-$CH_2$-, Act-O-$CH_2$-, aminomethyl, Prot-N($R^H$)-$CH_2$-, Act-N($R^H$)-$CH_2$-, carboxymethyl, sulphonomethyl, where Prot is a protection group for -OH, -SH, and -NH($R^H$), respectively, Act is an activation group for -OH, -SH, and -NH($R^H$), respectively, and $R^H$ is selected from hydrogen and $C_{1-6}$-alkyl;

(i) $R^{2*}$ and $R^{4*}$ together designate a biradical selected from -O-, -($CR^*R^*$)$_{r+s+1}$-, -($CR^*R^*$)$_r$-O-($CR^*R^*$)$_s$-, -($CR^*R^*$)$_r$-S-($CR^*R^*$)$_s$-, -($CR^*R^*$)$_r$-N($R^*$)-($CR^*R^*$)$_s$-, -O-($CR^*R^*$)$_{r+s}$-O-, -S-($CR^*R^*$)$_{r+s}$-O-, -O-($CR^*R^*$)$_{r+s}$-S-, -N($R^*$)-($CR^*R^*$)$_{r+s}$-O-, -O-($CR^*R^*$)$_{r+s}$-N($R^*$)-, -S-($CR^*R^*$)$_{r+s}$-S-, -N($R^*$)-($CR^*R^*$)$_{r+s}$-N($R^*$)-, -N($R^*$)-($CR^*R^*$)$_{r+s}$-S-, and -S-($CR^*R^*$)$_{r+s}$-N($R^*$)-;

(ii) $R^2$ and $R^3$ together designate a biradical selected from -O-, -($CR^*R^*$)$_{r+s}$-, -($CR^*R^*$)$_r$-O-($CR^*R^*$)$_s$-, -($CR^*R^*$)$_r$-S-($CR^*R^*$)$_s$-, and -($CR^*R^*$)$_r$-N($R^*$)-($CR^*R^*$)$_s$-;

(iii) $R^{2*}$ and $R^3$ together designate a biradical selected from -O-, -($CR^*R^*$)$_{r+s}$-, -($CR^*R^*$)$_r$-O-($CR^*R^*$)$_s$-, -($CR^*R^*$)$_r$-S-($CR^*R^*$)$_s$-, and -($CR^*R^*$)$_r$-N($R^*$)-($CR^*R^*$)$_s$-;

(iv) $R^3$ and $R^{4*}$ together designate a biradical selected from -($CR^*R^*$)$_r$-O-($CR^*R^*$)$_s$-, -($CR^*R^*$)$_r$-S-($CR^*R^*$)$_s$-, and -($CR^*R^*$)$_r$-N($R^*$)-($CR^*R^*$)$_s$-;

(v) $R^3$ and $R^5$ together designate a biradical selected from -($CR^*R^*$)$_r$-O-($CR^*R^*$)$_s$-, -($CR^*R^*$)$_r$-S-($CR^*R^*$)$_s$-, and -($CR^*R^*$)$_r$-N($R^*$)-($CR^*R^*$)$_s$-; or

(vi) $R^{1*}$ and $R^{4*}$ together designate a biradical selected from -$(CR^*R^*)_r$-O-$(CR^*R^*)_s$-, -$(CR^*R^*)_r$-S-$(CR^*R^*)_s$-, and -$(CR^*R^*)_r$-N($R^*$)-$(CR^*R^*)_s$-;

(vii) $R^{1*}$ and $R^{2*}$ together designate a biradical selected from -$(CR^*R^*)_r$-O-$(CR^*R^*)_s$-, -$(CR^*R^*)_r$-S-$(CR^*R^*)_s$-, and -$(CR^*R^*)_r$-N($R^*$)-$(CR^*R^*)_s$-;

wherein each $R^*$ is independently selected from hydrogen, halogen, azido, cyano, nitro, hydroxy, mercapto, amino, mono- or di($C_{1-6}$-alkyl)amino, optionally substituted $C_{1-6}$-alkoxy, optionally substituted $C_{1-6}$-alkyl, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, and/or two adjacent (non-geminal) $R^*$ may together designate a double bond, and each of r and s is 0-3 with the proviso that the sum r+s is 1-4;

each of the substituents $R^{1*}$, $R^2$, $R^{2*}$, $R^3$, $R^{4*}$, $R^5$, and $R^{5*}$, which are not involved in Q, $Q^*$ or the biradical, is independently selected from hydrogen, optionally substituted $C_{1-12}$-alkyl, optionally substituted $C_{2-12}$-alkenyl, optionally substituted $C_{2-12}$-alkynyl, hydroxy, $C_{1-12}$-alkoxy, $C_{2-12}$-alkenyloxy, carboxy, $C_{1-12}$-alkoxycarbonyl, $C_{1-12}$-alkylcarbonyl, formyl, aryl, aryloxy-carbonyl, aryloxy, arylcarbonyl, heteroaryl, heteroaryloxy-carbonyl, heteroaryloxy, heteroarylcarbonyl, amino, mono- and di($C_{1-6}$-alkyl)amino, carbamoyl, mono- and di($C_{1-6}$-alkyl)-amino-carbonyl, amino-$C_{1-6}$-alkyl-aminocarbonyl, mono- and di($C_{1-6}$-alkyl)amino-$C_{1-6}$-alkyl-aminocarbonyl, $C_{1-6}$-alkyl-carbonylamino, carbamido, $C_{1-6}$-alkanoyloxy, sulphono, $C_{1-6}$-alkylsulphonyloxy, nitro, azido, sulphanyl, $C_{1-6}$-alkylthio, halogen, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, where aryl and heteroaryl may be optionally substituted, and where two geminal substituents together may designate oxo, thioxo, imino, or optionally substituted methylene, or together may form a spiro biradical consisting of a 1-5 carbon atom(s) alkylene chain which is optionally interrupted and/or terminated by one or more heteroatoms/groups selected from -O-, -S-, and -($NR^N$)- where $R^N$ is selected from hydrogen and $C_{1-4}$-alkyl, and where two adjacent (non-geminal) substituents may designate an additional bond resulting in a double bond; and $R^{N*}$, when present and not involved in a biradical, is selected from hydrogen and $C_{1-4}$-alkyl;

and basic salts and acid addition salts thereof;

with the first proviso that,

(i) $R^2$ and $R^3$ do not together designate a biradical selected from -O-$CH_2$-$CH_2$- and -O-$CH_2$-$CH_2$-$CH_2$-; and

(ii) $R^3$ and $R^5$ do not together designate a biradical selected from -$CH_2$-$CH_2$-, -O-$CH_2$-, and -O-Si($^i$Pr)$_2$-O-Si($^i$Pr)$_2$-O-;

and with the second proviso that any chemical group (including any nucleobase), which is reactive under the conditions prevailing in oligonucleotide synthesis, is optionally functional group protected.

The present invention also relates to the use of the nucleoside analogues (LNAs) for the preparation of oligomers, and the use of the oligomers as well as the nucleoside analogues (LNAs) in diagnostics, molecular biology research, and in therapy.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B illustrate known conformationally restricted nucleotides.

Figure 2 illustrates nucleotide/nucleoside analogues of the invention.

Figure 3 illustrates the performance of LNA modified oligonucleotides in the sequence specific capture of PCR amplicons.

Figures 4A and 4B illustrate that LNA modified oligonucleotides are able to capture its cognate PCR amplicon by strand invasion.

Figure 5 illustrates that LNA modified oligonucleotides, immobilised on a solid surface, function efficiently in the sequence specific capture of a PCR amplicon.

Figure 6 illustrates that LNA modified oligonucleotides can act as substrates for T4 polynucleotide kinase.

Figure 7 illustrates that LNA modified oligonucleotides can function as primers for nucleic acid polymerases.

Figure 8 illustrates that LNA modified oligonucleotides can functions as primers in target amplification processes.

Figure 9 illustrates that LNA modified oligonucleotides carrying a 5´anthraquinone can be covalently immobilised on a solid support by irradiation and that the immobilised oligomer is efficient in the capture of a complementary DNA oligo.

Figure 10 illustrates that LNA-thymidine-5'-triphosphate (LNA-TTP) can act as a substrate for terminal deoxynucleotidyl transferase (TdT).

Figure 11 illustrates hybridisation and detection on an array with different LNA modified Cy3-labelled 8mers.

Figures 12 and 13 illustrate hybridisation and detection of end mismatches on an array with LNA modified Cy3-labelled 8mers.

Figure 14 illustrates blockade by LNA of [D-Ala2]deltorphin-induced antinociception in the warm water tail flick test in conscious rats.

Figures 15A, 15B, and 15C illustrate Hybridization and detection of end mismatches on an array with AT and all LNA modified Cy3-labelled 8mers.

Figures 16 and 17 illustrate that LNA can be delivered to living human MCF-7 breast cancer cells.

Figures 18 and 19 illustrate the use of [$\alpha^{33}$P] ddNTP's and ThermoSequenase™ DNA Polymerase to sequence DNA templates containing LNA T monomers.

Figures 20 and 21 illustrate that exonuclease free Klenow fragment DNA polymerase I can incorporate LNA Adenosine, Cytosine, Guanosine and Uridine-5'-triphosphates into a DNA strand.

Figure 22 illustrates the ability of terminal deoxynucleotidyl transferase (TdT) to tail LNA modified oligonucleotides.

Figures 23A and 23B illustrate that fully mixed LNA monomers can be used to significantly increase the performance of immobilised biotinylated-DNA oligos in the sequence specific capture of PCR amplicons.

Figures 24 to 41 illustrates possible synthetic routes towards the LNA monomers of the invention.

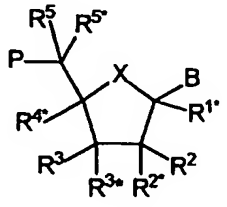
## DETAILED DESCRIPTION OF THE INVENTION

When used herein, the term "LNA" (Locked Nucleoside Analogues) refers to the bi- and tri-cyclic nucleoside analogues of the invention, either incorporated in the oligomer of the invention (general formula I) or as discrete chemical species (general formula II). The term "monomeric LNA" specifically refers to the latter case.

**Oligomers and nucleoside analogues**

As mentioned above, the present invention *i.a.* relates to novel oligomers (oligonucleotides) comprising one or more bi-, tri-, or polycyclic nucleoside analogues (hereinafter termed "LNA"). It has been found that the incorporation of such LNAs in place of, or as a supplement to, *e.g.*, known nucleosides confer interesting and highly useful properties to an oligonucleotide. Bi- and tricyclic, especially bicyclic, LNAs seem especially interesting within the scope of the present invention.

Each of the possible LNAs incorporated in an oligomer (oligonucleotide) has the general formula I




I

wherein X is selected from -O- (the furanose motif), -S-, -N($R^{N*}$)-, -C($R^6R^{6*}$)-, -O-C($R^7R^{7*}$)-, -C($R^6R^{6*}$)-O-, -S-C($R^7R^{7*}$)-, -C($R^6R^{6*}$)-S-, -N($R^{N*}$)-C($R^7R^{7*}$)-, -C($R^6R^{6*}$)-N($R^{N*}$)-, and -C($R^6R^{6*}$)-C($R^7R^{7*}$)-, where $R^6$, $R^{6*}$, $R^7$, $R^{7*}$, and $R^{N*}$ are as defined further below. Thus, the LNAs incorporated in the oligomer may comprise an either 5- or 6-membered ring as an essential part of the bi-, tri-, or polycyclic structure. It is believed that 5-membered rings (X = -O-, -S-, -N($R^{N*}$)-, -C($R^6R^{6*}$)-) are especially interesting in that they are able to occupy essentially the same conformations (however locked by the introduction of one or more biradicals (see below)) as the native furanose ring of a naturally occurring nucleoside. Among the possible 5-membered rings, the situations where X designates -O-, -S-, and -N($R^{N*}$)- seem especially interesting, and the situation where X is -O- appears to be particularly interesting.

The substituent B may designate a group which, when the oligomer is complexing with DNA or RNA, is able to interact (*e.g.* by hydrogen bonding or covalent bonding or electronic interaction) with DNA or RNA, especially nucleobases of DNA or RNA. Alternatively, the substituent B may designate a group which acts as a label or a reporter, or the substituent B may designate a group (*e.g.* hydrogen) which is expected to have little or no interactions with DNA or RNA. Thus, the substituent B is preferably selected from hydrogen, hydroxy, optionally substituted $C_{1-4}$-alkoxy, optionally substituted $C_{1-4}$-alkyl, optionally substituted $C_{1-4}$-acyloxy, nucleobases, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands.

In the present context, the terms "nucleobase" covers naturally occurring nucleobases as well as non-naturally occurring nucleobases. It should be clear to the person skilled in the art that various nucleobases which previously have been considered "non-naturally occurring" have subsequently been found in nature. Thus, "nucleobase" includes not only the known purine and pyrimidine heterocycles, but also heterocyclic analogues and tautomers thereof. Illustrative examples of nucleobases are adenine, guanine, thymine, cytosine, uracil, purine, xanthine, diaminopurine, 8-oxo-$N^6$-methyladenine, 7-deazaxanthine, 7-deazaguanine, $N^4$,$N^4$-ethanocytosin, $N^6$,$N^6$-ethano-2,6-diaminopurine, 5-methylcytosine, 5-($C^3$-$C^6$)-alkynylcytosine, 5-fluorouracil, 5-bromouracil, pseudoisocytosine, 2-hydroxy-5-methyl-4-triazolopyridin, isocytosine,

isoguanin, inosine and the "non-naturally occurring" nucleobases described in Benner et al., U.S. Pat No. 5,432,272. The term "nucleobase" is intended to cover every and all of these examples as well as analogues and tautomers thereof. Especially interesting nucleobases are adenine, guanine, thymine, cytosine, and uracil, which are considered as the naturally occurring nucleobases in relation to therapeutic and diagnostic application in humans.

When used herein, the term "DNA intercalator" means a group which can intercalate into a DNA or RNA helix, duplex or triplex. Examples of functional parts of DNA intercalators are acridines, anthracene, quinones such as anthraquinone, indole, quinoline, isoquinoline, dihydroquinones, anthracyclines, tetracyclines, methylene blue, anthracyclinone, psoralens, coumarins, ethidium-halides, dynemicin, metal complexes such as 1,10-phenanthroline-copper, tris(4,7-diphenyl-1,10-phenanthroline)ruthenium-cobalt-enediynes such as calcheamicin, porphyrins, distamycin, netropcin, viologen, daunomycin. Especially interesting examples are acridines, quinones such as anthraquinone, methylene blue, psoralens, coumarins, and ethidium-halides.

In the present context, the term "photochemically active groups" covers compounds which are able to undergo chemical reactions upon irradiation with light. Illustrative examples of functional groups hereof are quinones, especially 6-methyl-1,4-naphtoquinone, anthraquinone, naphtoquinone, and 1,4-dimethyl-anthraquinone, diazirines, aromatic azides, benzophenones, psoralens, diazo compounds, and diazirino compounds.

In the present context "thermochemically reactive group" is defined as a functional group which is able to undergo thermochemically-induced covalent bond formation with other groups. Illustrative examples of functional parts thermochemically reactive groups are carboxylic acids, carboxylic acid esters such as activated esters, carboxylic acid halides such as acid fluorides, acid chlorides, acid bromide, and acid iodides, carboxylic acid azides, carboxylic acid hydrazides, sulfonic acids, sulfonic acid esters, sulfonic acid halides, semicarbazides, thiosemicarbazides, aldehydes, ketones, primary alkohols, secondary alkohols, tertiary alkohols, phenols, alkyl halides, thiols, disulphides, primary amines, secondary amines, tertiary amines, hydrazines, epoxides, maleimides, and boronic acid derivatives.

In the present context, the term "chelating group" means a molecule that contains more than one binding site and frequently binds to another molecule, atom or ion through more than one binding site at the same time. Examples of functional parts of chelating groups are iminodiacetic acid, nitrilotriacetic acid, ethylenediamine tetraacetic acid (EDTA), aminophosphonic acid, etc.

In the present context, the term "reporter group" means a group which is detectable either by itself or as a part of an detection series. Examples of functional parts of reporter groups are biotin, digoxigenin, fluorescent groups (groups which are able to absorb electromagnetic radiation, *e.g.* light or X-rays, of a certain wavelength, and which subsequently reemits the energy absorbed as radiation of longer wavelength; illustrative examples are dansyl (5-dimethylamino)-1-naphthalenesulfonyl), DOXYL (N-oxyl-4,4-dimethyloxazolidine), PROXYL (N-oxyl-2,2,5,5-tetramethylpyrrolidine), TEMPO (N-oxyl-2,2,6,6-tetramethylpiperidine), dinitrophenyl, acridines, coumarins, Cy3 and Cy5 (trademarks for Biological Detection Systems, Inc.), erytrosine, coumaric acid, umbelliferone, texas red, rhodamine, tetramethyl rhodamine, Rox, 7-nitrobenzo-2-oxa-1-diazole (NBD), pyrene, fluorescein, Europium, Ruthenium, Samarium, and other rare earth metals), radioisotopic labels, chemiluminescence labels (labels that are detectable via the emission of light during a chemical reaction), spin labels (a free radical (*e.g.* substituted organic nitroxides) or other paramagnetic probes (*e.g.* $Cu^{2+}$, $Mg^{2+}$) bound to a biological molecule being detectable by the use of electron spin resonance spectroscopy), enzymes (such as peroxidases, alkaline phosphatases, β-galactosidases, and glycose oxidases), antigens, antibodies, haptens (groups which are able to combine with an antibody, but which cannot initiate an immune response by itself, such as peptides and steroid hormones), carrier systems for cell membrane penetration such as: fatty acid residues, steroid moieties (cholesteryl), vitamin A, vitamin D, vitamin E, folic acid peptides for specific receptors, groups for mediating endocytose, epidermal growth factor (EGF), bradykinin, and platelet derived growth factor (PDGF). Especially interesting examples are biotin, fluorescein, Texas Red, rhodamine, dinitrophenyl, digoxigenin, Ruthenium, Europium, Cy5, Cy3, etc.

In the present context "ligand" means something which binds. Ligands can comprise functional groups such as: aromatic groups (such as benzene, pyridine, naphtalene,

anthracene, and phenanthrene), heteroaromatic groups (such as thiophene, furan, tetrahydrofuran, pyridine, dioxane, and pyrimidine), carboxylic acids, carboxylic acid esters, carboxylic acid halides, carboxylic acid azides, carboxylic acid hydrazides, sulfonic acids, sulfonic acid esters, sulfonic acid halides, semicarbazides, thiosemicarbazides, aldehydes, ketones, primary alcohols, secondary alcohols, tertiary alcohols, phenols, alkyl halides, thiols, disulphides, primary amines, secondary amines, tertiary amines, hydrazines, epoxides, maleimides, $C_1$-$C_{20}$ alkyl groups optionally interrupted or terminated with one or more heteroatoms such as oxygen atoms, nitrogen atoms, and/or sulphur atoms, optionally containing aromatic or mono/polyunsaturated hydrocarbons, polyoxyethylene such as polyethylene glycol, oligo/polyamides such as poly-β-alanine, polyglycine, polylysine, peptides, oligo/polysaccharides, oligo/polyphosphates, toxins, antibiotics, cell poisons, and steroids, and also "affinity ligands", *i.e.* functional groups or biomolecules that have a specific affinity for sites on particular proteins, antibodies, poly- and oligosaccharides, and other biomolecules.

It will be clear for the person skilled in the art that the above-mentioned specific examples under DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands correspond to the "active/functional" part of the groups in question. For the person skilled in the art it is furthermore clear that DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands are typically represented in the form M-K- where M is the "active/functional" part of the group in question and where K is a spacer through which the "active/functional" part is attached to the 5- or 6-membered ring. Thus, it should be understood that the group B, in the case where B is selected from DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, has the form M-K-, where M is the "active/functional" part of the DNA intercalator, photochemically active group, thermochemically active group, chelating group, reporter group, and ligand, respectively, and where K is an optional spacer comprising 1-50 atoms, preferably 1-30 atoms, in particular 1-15 atoms, between the 5- or 6-membered ring and the "active/functional" part.

In the present context, the term "spacer" means a thermochemically and photochemically non-active distance-making group and is used to join two or more different moieties of the types defined above. Spacers are selected on the basis of a variety of characteristics including their hydrophobicity , hydrophilicity, molecular flexibility and length (*e.g.* see Hermanson et. al., "Immobilized Affinity Ligand Techniques", Academic Press, San Diego, California (1992), p. 137-ff). Generally, the length of the spacers are less than or about 400 Å, in some applications preferably less than 100 Å. The spacer, thus, comprises a chain of carbon atoms optionally interrupted or terminated with one or more heteroatoms, such as oxygen atoms, nitrogen atoms, and/or sulphur atoms. Thus, the spacer K may comprise one or more amide, ester, amino, ether, and/or thioether functionalities, and optionally aromatic or mono/polyunsaturated hydrocarbons, polyoxyethylene such as polyethylene glycol, oligo/polyamides such as poly-β-alanine, polyglycine, polylysine, and peptides in general, oligosaccharides, oligo/polyphosphates. Moreover the spacer may consist of combined units thereof. The length of the spacer may vary, taking into consideration the desired or necessary positioning and spatial orientation of the "active/functional" part of the group in question in relation to the 5- or 6-membered ring. In particularly interesting embodiments, the spacer includes a chemically cleavable group. Examples of such chemically cleavable groups include disulphide groups cleavable under reductive conditions, peptide fragments cleavable by peptidases, etc.

In one embodiment of the present invention, K designates a single bond so that the "active/functional" part of the group in question is attached directly to the 5- or 6-membered ring.

In a preferred embodiment, the substituent B in the general formulae I and II is preferably selected from nucleobases, in particular from adenine, guanine, thymine, cytosine and urasil.

In the oligomers of the present invention (formula I), P designates the radical position for an internucleoside linkage to a succeeding monomer, or a 5'-terminal group. The first possibility applies when the LNA in question is not the 5'-terminal "monomer", whereas the latter possibility applies when the LNA in question is the 5'-terminal "monomer". It should be understood (which also will be clear from the definition of

internucleoside linkage and 5'-terminal group further below) that such an internucleoside linkage or 5'-terminal group may include the substituent $R^5$ (or equally applicable: the substituent $R^{5*}$) thereby forming a double bond to the group P. (5'-Terminal refers to the position corresponding to the 5' carbon atom of a ribose moiety in a nucleoside.)

On the other hand, an internucleoside linkage to a preceding monomer or a 3'-terminal group ($P^*$) may originate from the positions defined by one of the substituents $R^2$, $R^{2*}$, $R^3$, and $R^{3*}$, preferably from the positions defined by one of the substituents $R^3$ and $R^{3*}$. Analogously, the first possibility applies where the LNA in question is not the 3'-terminal "monomer", whereas the latter possibility applies when the LNA in question is the 3'-terminal "monomer". (3'-Terminal refers to the position corresponding to the 3' carbon atom of a ribose moiety in a nucleoside.)

In the present context, the term "monomer" relates to naturally occurring nucleosides, non-naturally occurring nucleosides, PNAs, etc. as well as LNAs. Thus, the term "succeeding monomer" relates to the neighbouring monomer in the 5'-terminal direction and the "preceding monomer" relates to the neighbouring monomer in the 3'-terminal direction. Such succeeding and preceding monomers, seen from the position of an LNA monomer, may be naturally occurring nucleosides or non-naturally occurring nucleosides, or even further LNA monomers.

Consequently, in the present context (as can be derived from the definitions above), the term "oligomer" means an oligonucleotide modified by the incorporation of one or more LNA(s).

The crucial part of the present invention is the presence of one or more rings fused to the 5- or 6-membered ring illustrated with the general formula I. Thus, one or two pairs of non-geminal substituents selected from the present substituents of $R^{1*}$, $R^{4*}$, $R^5$, $R^{5*}$, $R^6$, $R^{6*}$, $R^7$, $R^{7*}$, $R^{N*}$, and the ones of $R^2$, $R^{2*}$, $R^3$, and $R^{3*}$ not designating $P^*$ each designates a biradical consisting of 1-8 groups/atoms, preferably 1-4 groups/atoms, independently selected from $-C(R^aR^b)-$, $-C(R^a)=C(R^a)-$, $-C(R^a)=N-$, $-O-$, $-Si(R^a)_2-$, $-S-$, $-SO_2-$, $-N(R^a)-$, and $>C=Z$. (The term "present" indicates that the

existence of some of the substituents, *i.e.* $R^6$, $R^{6*}$, $R^7$, $R^{7*}$, $R^{N*}$, is dependent on whether X includes such substituents.)

In the groups constituting the biradical(s), Z is selected from -O-, -S-, and -N($R^a$)-, and $R^a$ and $R^b$ each is independently selected from hydrogen, optionally substituted $C_{1-12}$-alkyl, optionally substituted $C_{2-12}$-alkenyl, optionally substituted $C_{2-12}$-alkynyl, hydroxy, $C_{1-12}$-alkoxy, $C_{2-12}$-alkenyloxy, carboxy, $C_{1-12}$-alkoxycarbonyl, $C_{1-12}$-alkylcarbonyl, formyl, aryl, aryloxy-carbonyl, aryloxy, arylcarbonyl, heteroaryl, heteroaryloxy-carbonyl, heteroaryloxy, heteroarylcarbonyl, amino, mono- and di($C_{1-6}$-alkyl)amino, carbamoyl, mono- and di($C_{1-6}$-alkyl)-amino-carbonyl, amino-$C_{1-6}$-alkyl-aminocarbonyl, mono- and di($C_{1-6}$-alkyl)amino-$C_{1-6}$-alkyl-aminocarbonyl, $C_{1-6}$-alkyl-carbonylamino, carbamido, $C_{1-6}$-alkanoyloxy, sulphono, $C_{1-6}$-alkylsulphonyloxy, nitro, azido, sulphanyl, $C_{1-6}$-alkylthio, halogen, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands (where the latter groups may include a spacer as defined for the substituent B), where aryl and heteroaryl may be optionally substituted. Moreover, two geminal substituents $R^a$ and $R^b$ together may designate optionally substituted methylene (=$CH_2$ optionally substituted one or two times with substituents as defined as optional substituents for aryl), and two non-geminal or geminal substituents selected from $R^a$, $R^b$, and any of the substituents $R^{1*}$, $R^2$, $R^{2*}$, $R^3$, $R^{3*}$, $R^{4*}$, $R^5$, $R^{5*}$, $R^6$ and $R^{6*}$, $R^7$, and $R^{7*}$ which are present and not involved in P, $P^*$ or the biradical(s) may together form an associated biradical selected from biradicals of the same kind as defined before. It will be clear that each of the pair(s) of non-geminal substituents thereby forms a mono- or bicyclic entity together with (i) the atoms to which the non-geminal substituents are bound and (ii) any intervening atoms.

It is believed that biradicals which are bound to the ring atoms of the 5- or 6-membered rings are preferred in that inclusion of the substituents $R^5$ and $R^{5*}$ may cause an undesired sterical interaction with internucleoside linkage. Thus, it is preferred that the one or two pairs of non-geminal substituents, which are constituting one or two biradical(s), respectively, are selected from the present substituents of $R^{1*}$, $R^{4*}$, $R^6$, $R^{6*}$, $R^7$, $R^{7*}$, $R^{N*}$, and the ones of $R^2$, $R^{2*}$, $R^3$, and $R^{3*}$ not designating $P^*$.

Preferably, the LNAs incorporated in the oligomers comprise only one biradical constituted by a pair of (two) non-geminal substituents. In particular, it is preferred that $R^{3*}$ designates $P^*$ and that the biradical is formed between $R^{2*}$ and $R^{4*}$ or $R^2$ and $R^3$.

This being said, it should be understood (especially with due consideration of the known bi- and tricyclic nucleoside analogues - see "Background of the Invention") that the present invention does not relate to oligomers comprising the following bi- or tricyclic nucleosides analogues:

(i) $R^2$ and $R^3$ together designate a biradical selected from $-O-CH_2-CH_2-$ and $-O-CH_2-CH_2-CH_2-$ when LNA is a bicyclic nucleoside analogue;

(ii) $R^3$ and $R^5$ together designate a biradical selected from $-CH_2-CH_2-$, $-O-CH_2-$, when LNA is a bicyclic nucleoside analogue;

(iii) $R^3$, $R^5$, and $R^{5*}$ together designate a triradical $-CH_2-CH(-)-CH_2-$ when LNA is a tricyclic nucleoside analogue;

(iv) $R^{1*}$ and $R^{6*}$ together designate a biradical $-CH_2-$ when LNA is a bicyclic nucleoside analogue; or

(v) $R^{4*}$ and $R^{6*}$ together designate a biradical $-CH_2-$ when LNA is a bicyclic nucleoside analogue;

except where such bi- or tricyclic nucleoside analogues are combined with one or more of the novel LNAs defined herein.

In the present context, *i.e.* in the present description and claims, the orientation of the biradicals are so that the left-hand side represents the substituent with the lowest number and the right-hand side represents the substituent with the highest number, thus, when $R^3$ and $R^5$ together designate a biradical "$-O-CH_2-$", it is understood that the oxygen atom represents $R^3$, thus the oxygen atom is *e.g.* attached to the position of $R^3$, and the methylene group represents $R^5$.

Considering the numerous interesting possibilities for the structure of the biradical(s) in LNA(s) incorporated in oligomers according to the invention, it is believed that the biradical(s) constituted by pair(s) of non-geminal substituents preferably is/are selected

from -(CR*R*)$_r$-Y-(CR*R*)$_s$-, -(CR*R*)$_r$-Y-(CR*R*)$_s$-Y-, -Y-(CR*R*)$_{r+s}$-Y-, -Y-(CR*R*)$_r$-Y-(CR*R*)$_s$-, -(CR*R*)$_{r+s}$-, -Y-, -Y-Y-, wherein each Y is independently selected from -O-, -S-, -Si(R*)$_2$-, -N(R*)-, >C=O, -C(=O)-N(R*)-, and -N(R*)-C(=O)-, each R* is independently selected from hydrogen, halogen, azido, cyano, nitro, hydroxy, mercapto, amino, mono- or di($C_{1-6}$-alkyl)amino, optionally substituted $C_{1-6}$-alkoxy, optionally substituted $C_{1-6}$-alkyl, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, and/or two adjacent (non-geminal) R* may together designate a double bond; and each of r and s is 0-4 with the proviso that the sum r+s is 1-5. Particularly interesting situations are those wherein each biradical is independently selected from -Y-, -(CR*R*)$_{r+s}$-, -(CR*R*)$_r$-Y-(CR*R*)$_s$-, and -Y-(CR*R*)$_{r+s}$-Y-, wherein and each of r and s is 0-3 with the proviso that the sum r+s is 1-4.

Considering the positioning of the biradical in the LNA(s), it is believed (based on the preliminary findings (see the examples)) that the following situations are especially interesting, namely where: $R^{2*}$ and $R^{4*}$ together designate a biradical selected from -Y-, -(CR*R*)$_{r+s+1}$-, -(CR*R*)$_r$-Y-(CR*R*)$_s$-, and -Y-(CR*R*)$_{r+s}$-Y-; $R^2$ and $R^3$ together designate a biradical selected from -Y-, -(CR*R*)$_{r+s}$-, -(CR*R*)$_r$-Y-(CR*R*)$_s$-, and -Y-(CR*R*)$_{r+s}$-Y-; $R^{2*}$ and $R^3$ together designate a biradical selected from -Y-, -(CR*R*)$_{r+s}$-, -(CR*R*)$_r$-Y-(CR*R*)$_s$-, and -Y-(CR*R*)$_{r+s}$-Y-; $R^3$ and $R^{4*}$ together designate a biradical selected from -Y-, -(CR*R*)$_{r+s}$-, -(CR*R*)$_r$-Y-(CR*R*)$_s$-, and -Y-(CR*R*)$_{r+s}$-Y-; $R^3$ and $R^5$ together designate a biradical selected from -Y'-, -(CR*R*)$_{r+s+1}$-, -(CR*R*)$_r$-Y-(CR*R*)$_s$-, and -Y-(CR*R*)$_{r+s}$-Y-; $R^{1*}$ and $R^{4*}$ together designate a biradical selected from -Y'-, -(CR*R*)$_{r+s+1}$-, -(CR*R*)$_r$-Y-(CR*R*)$_s$-, and -Y-(CR*R*)$_{r+s}$-NR*-; or where $R^{1*}$ and $R^{2*}$ together designate a biradical selected from -Y-, -(CR*R*)$_{r+s}$-, -(CR*R*)$_r$-Y-(CR*R*)$_s$-, and -Y-(CR*R*)$_{r+s}$-Y-; wherein each of r and s is 0-3 with the proviso that the sum r+s is 1-4, Y is as defined above, and where Y' is selected from -NR*-C(=O)- and -C(=O)-NR*-.

Particularly interesting oligomers are those wherein one of the following criteria applies for at least one LNA in an oligomer: $R^{2*}$ and $R^{4*}$ together designate a biradical selected from -O-, -S-, -N(R*)-, -(CR*R*)$_{r+s+1}$-, -(CR*R*)$_r$-O-(CR*R*)$_s$-, -(CR*R*)$_r$-S-(CR*R*)$_s$-, -(CR*R*)$_r$-N(R*)-(CR*R*)$_s$-, -O-(CR*R*)$_{r+s}$-O-, -S-(CR*R*)$_{r+s}$-O-, -O-(CR*R*)$_{r+s}$-S-, -N(R*)-(CR*R*)$_{r+s}$-O-, -O-(CR*R*)$_{r+s}$-N(R*)-, -S-(CR*R*)$_{r+s}$-S-, -N(R*)-(CR*R*)$_{r+s}$-N(R*)-, -N(R*)-

$(CR^*R^*)_{r+s}$-S-, and -S-$(CR^*R^*)_{r+s}$-N($R^*$)-; $R^2$ and $R^3$ together designate a biradical selected from -O-, -$(CR^*R^*)_{r+s}$-, -$(CR^*R^*)_r$-O-$(CR^*R^*)_s$-, -$(CR^*R^*)_r$-S-$(CR^*R^*)_s$-, and -$(CR^*R^*)_r$-N($R^*$)-$(CR^*R^*)_s$-; $R^{2*}$ and $R^3$ together designate a biradical selected from -O-, -$(CR^*R^*)_{r+s}$-, -$(CR^*R^*)_r$-O-$(CR^*R^*)_s$-, -$(CR^*R^*)_r$-S-$(CR^*R^*)_s$-, and -$(CR^*R^*)_r$-N($R^*$)-$(CR^*R^*)_s$-; $R^3$ and $R^{4*}$ together designate a biradical selected from -$(CR^*R^*)_r$-O-$(CR^*R^*)_s$-, -$(CR^*R^*)_r$-S-$(CR^*R^*)_s$-, and -$(CR^*R^*)_r$-N($R^*$)-$(CR^*R^*)_s$-; $R^3$ and $R^5$ together designate a biradical selected from -$(CR^*R^*)_r$-O-$(CR^*R^*)_s$-, -$(CR^*R^*)_r$-S-$(CR^*R^*)_s$-, and -$(CR^*R^*)_r$-N($R^*$)-$(CR^*R^*)_s$-; $R^{1*}$ and $R^{4*}$ together designate a biradical selected from -$(CR^*R^*)_r$-O-$(CR^*R^*)_s$-, -$(CR^*R^*)_r$-S-$(CR^*R^*)_s$-, and -$(CR^*R^*)_r$-N($R^*$)-$(CR^*R^*)_s$-; or $R^{1*}$ and $R^{2*}$ together designate a biradical selected from -$(CR^*R^*)_r$-O-$(CR^*R^*)_s$-, -$(CR^*R^*)_r$-S-$(CR^*R^*)_s$-, and -$(CR^*R^*)_r$-N($R^*$)-$(CR^*R^*)_s$-; wherein each of r and s is 0-3 with the proviso that the sum r+s is 1-4, and where $R^H$ designates hydrogen or $C_{1-4}$-alkyl.

It is furthermore preferred that one $R^*$ is selected from hydrogen, hydroxy, optionally substituted $C_{1-6}$-alkoxy, optionally substituted $C_{1-6}$-alkyl, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, and any remaining substituents $R^*$ are hydrogen.

In one preferred embodiment, one group $R^*$ in the biradical of at least one LNA is selected from DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands (where the latter groups may include a spacer as defined for the substituent B).

With respect to the substituents $R^{1*}$, $R^2$, $R^{2*}$, $R^3$, $R^{4*}$, $R^5$, $R^{5*}$, $R^6$ and $R^{6*}$, $R^7$, and $R^{7*}$, which are present and not involved in P, P* or the biradical(s), these are independently selected from hydrogen, optionally substituted $C_{1-12}$-alkyl, optionally substituted $C_{2-12}$-alkenyl, optionally substituted $C_{2-12}$-alkynyl, hydroxy, $C_{1-12}$-alkoxy, $C_{2-12}$-alkenyloxy, carboxy, $C_{1-12}$-alkoxycarbonyl, $C_{1-12}$-alkylcarbonyl, formyl, aryl, aryloxy-carbonyl, aryloxy, arylcarbonyl, heteroaryl, heteroaryloxy-carbonyl, heteroaryloxy, heteroarylcarbonyl, amino, mono- and di($C_{1-6}$-alkyl)amino, carbamoyl, mono- and di($C_{1-6}$-alkyl)-amino-carbonyl, amino-$C_{1-6}$-alkyl-aminocarbonyl, mono- and di($C_{1-6}$-alkyl)amino-$C_{1-6}$-alkyl-aminocarbonyl, $C_{1-6}$-alkyl-carbonylamino, carbamido, $C_{1-6}$-alkanoyloxy, sulphono, $C_{1-6}$-alkylsulphonyloxy, nitro, azido, sulphanyl, $C_{1-6}$-alkylthio, halogen, DNA intercalators, photochemically active groups, thermochemically active groups,

chelating groups, reporter groups, and ligands (where the latter groups may include a spacer as defined for the substituent B), where aryl and heteroaryl may be optionally substituted, and where two geminal substituents together may designate oxo, thioxo, imino, or optionally substituted methylene, or together may form a spiro biradical consisting of a 1-5 carbon atom(s) alkylene chain which is optionally interrupted and/or terminated by one or more heteroatoms/groups selected from -O-, -S-, and -($NR^N$)- where $R^N$ is selected from hydrogen and $C_{1-4}$-alkyl, and where two adjacent (non-geminal) substituents may designate an additional bond resulting in a double bond; and $R^{N*}$, when present and not involved in a biradical, is selected from hydrogen and $C_{1-4}$-alkyl.

Preferably, each of the substituents $R^{1*}$, $R^2$, $R^{2*}$, $R^3$, $R^{3*}$, $R^{4*}$, $R^5$, $R^{5*}$, $R^6$, $R^{6*}$, $R^7$, and $R^{7*}$ of the LNA(s), which are present and not involved in P, $P^*$ or the biradical(s), is independently selected from hydrogen, optionally substituted $C_{1-6}$-alkyl, optionally substituted $C_{2-6}$-alkenyl, hydroxy, $C_{1-6}$-alkoxy, $C_{2-6}$-alkenyloxy, carboxy, $C_{1-6}$-alkoxycarbonyl, $C_{1-6}$-alkylcarbonyl, formyl, amino, mono- and di($C_{1-6}$-alkyl)amino, carbamoyl, mono- and di($C_{1-6}$-alkyl)-amino-carbonyl, $C_{1-6}$-alkyl-carbonylamino, carbamido, azido, $C_{1-6}$-alkanoyloxy, sulphono, sulphanyl, $C_{1-6}$-alkylthio, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, and halogen, where two geminal substituents together may designate oxo, and where $R^{N*}$, when present and not involved in a biradical, is selected from hydrogen and $C_{1-4}$-alkyl.

In a preferred embodiment of the present invention, X is selected from -O-, -S-, and -$NR^{N*}$-, in particular -O-, and each of the substituents $R^{1*}$, $R^2$, $R^{2*}$, $R^3$, $R^{3*}$, $R^{4*}$, $R^5$, $R^{5*}$, $R^6$, $R^{6*}$, $R^7$, and $R^{7*}$ of the LNA(s), which are present and not involved in P, $P^*$ or the biradical(s), designate hydrogen.

In an even more preferred embodiment of the present invention, $R^{2*}$ and $R^{4*}$ of an LNA incorporated into an oligomer together designate a biradical. Preferably, X is O, $R^2$ selected from hydrogen, hydroxy, and optionally substituted $C_{1-6}$-alkoxy, and $R^{1*}$, $R^3$, $R^5$, and $R^{5*}$ designate hydrogen, and, more specifically, the biradical is selected from -O-, -$(CH_2)_{0-1}$-O-$(CH_2)_{1-3}$-, -$(CH_2)_{0-1}$-S-$(CH_2)_{1-3}$-, -$(CH_2)_{0-1}$-N($R^N$)-$(CH_2)_{1-3}$-, and -$(CH_2)_{2-4}$-, in particular from -O-$CH_2$-, -S-$CH_2$-, and -$NR^H$-$CH_2$-. Generally, with due regard to the

results obtained so far, it is preferred that the biradical constituting $R^{2*}$ and $R^{4*}$ forms a two carbon atom bridge, *i.e.* the biradical forms a five membered ring with the furanose ring (X=O).

In another embodiment of the present invention, $R^2$ and $R^3$ of an LNA incorporated into an oligomer together designate a biradical. Preferably, X is O, $R^{2*}$ is selected from hydrogen, hydroxy, and optionally substituted $C_{1-6}$-alkoxy, and $R^{1*}$, $R^{4*}$, $R^5$, and $R^{5*}$ designate hydrogen, and, more specifically, the biradical is selected from $-(CH_2)_{0-1}-O-(CH_2)_{1-3}-$, $-(CH_2)_{0-1}-S-(CH_2)_{1-3}-$, $-(CH_2)_{0-1}-N(R^H)-(CH_2)_{1-3}-$ and $-(CH_2)_{1-4}-$, in particular from $-O-CH_2-$, $-S-CH_2-$, $-N(R^H)-CH_2-$. In the latter case, the amino and thio variants appears to be particularly interesting.

In a further embodiment of the present invention, $R^{2*}$ and $R^3$ of an LNA incorporated into an oligomer together designate a biradical. Preferably, X is O, $R^2$ is selected from hydrogen, hydroxy, and optionally substituted $C_{1-6}$-alkoxy, and $R^{1*}$, $R^{4*}$, $R^5$, and $R^{5*}$ designate hydrogen, and, more specifically, the biradical is selected from $-(CH_2)_{0-1}-O-(CH_2)_{1-3}-$ and $-(CH_2)_{2-4}-$.

In a further embodiment of the present invention, $R^3$ and $R^{4*}$ of an LNA incorporated into an oligomer together designate a biradical. Preferably, X is O, $R^{2*}$ selected from hydrogen, hydroxy, and optionally substituted $C_{1-6}$-alkoxy, and $R^{1*}$, $R^2$, $R^5$, and $R^{5*}$ designate hydrogen, and, more specifically, the biradical is $-(CH_2)_{0-2}-O-(CH_2)_{0-2}-$.

In a further embodiment of the present invention, $R^3$ and $R^{5*}$ of an LNA incorporated into an oligomer together designate a biradical. Preferably, X is O, $R^{2*}$ selected from hydrogen, hydroxy, and optionally substituted $C_{1-6}$-alkoxy, and $R^{1*}$, $R^2$, $R^4$, and $R^5$ designate hydrogen, and, more specifically, the biradical is selected from $-O-(CHR^*)_{2-3}-$ and $-(CHR^*)_{1-3}-O-(CHR^*)_{0-3}-$.

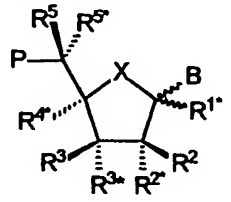
In a further embodiment of the present invention, $R^{1*}$ and $R^{4*}$ of an LNA incorporated into an oligomer together designate a biradical. Preferably, X is O, $R^{2*}$ selected from hydrogen, hydroxy, and optionally substituted $C_{1-6}$-alkoxy, and $R^2$, $R^3$, $R^5$, and $R^{5*}$ designate hydrogen, and, more specifically, the biradical is $-(CH_2)_{0-2}-O-(CH_2)_{0-2}-$.

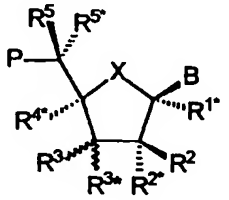
In these embodiments, it is furthermore preferred that at least one LNA incorporated in an oligomer includes a nucleobase (substituent B) selected from adenine and guanine. In particular, it is preferred that an oligomer have LNA incorporated therein both include at least one nucleobase selected from thymine, urasil and cytosine and at least one nucleobase selected from adenine and guanine. For LNA monomers, it is especially preferred that the nucleobase is selected from adenine and guanine.

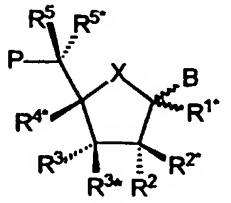
For these interesting embodiments, it is also preferred that the LNA(s) has/have the general formula Ia (see below).

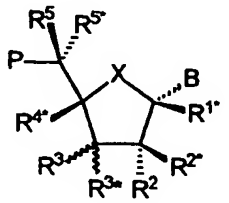
Within a variant of these interesting embodiments, all monomers of a oligonucleotide are LNA monomers.

As it will be evident from the general formula I (LNA(s) in an oligomer) (and the general formula II (monomeric LNA) - see below) and the definitions associated therewith, there may be one or several asymmetric carbon atoms present in the oligomers (and monomeric LNAs) depending on the nature of the substituents and possible biradicals, cf. below. The oligomers prepared according to the method of the invention, as well as the oligomers per se, are intended to include all stereoisomers arising from the presence of any and all isomers of the individual monomer fragments as well as mixtures thereof, including racemic mixtures. When considering the 5- or 6-membered ring, it is, however, believed that certain stereochemical configurations will be especially interesting, *e.g.* the following



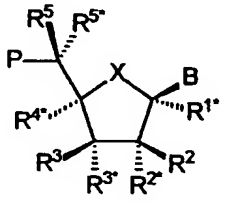



where the wavy lines represent the possibility of both diastereomers arising from the interchange of the two substituents in question.

An especially interesting stereoisomeric representation is the case where the LNA(s) has/have the following formula Ia



Ia

Also interesting as a separate aspect of the present invention is the variant of formula Ia where B is in the "α-configuration".

In these cases, as well as generally, $R^{3*}$ preferably designates $P^*$.

The oligomers according to the invention typically comprise 1-10000 LNA(s) of the general formula I (or of the more detailed general formula Ia) and 0-10000 nucleosides selected from naturally occurring nucleosides and nucleoside analogues. The sum of the number of nucleosides and the number of LNA(s) is at least 2, preferably at least 3, in particular at least 5, especially at least 7, such as in the range of 2-15000, preferably in the range of 2-100, such as 3-100, in particular in the range of 2-50, such as 3-50 or 5-50 or 7-50.

Preferably at least one LNA comprises a nucleobase as the substituent B.

In the present context, the term "nucleoside" means a glycoside of a heterocyclic base. The term "nucleoside" is used broadly as to include non-naturally occurring nucleosides, naturally occurring nucleosides as well as other nucleoside analogues. Illustrative examples of nucleosides are ribonucleosides comprising a ribose moiety as well as deoxyribonuclesides comprising a deoxyribose moiety. With respect to the bases of such nucleosides, it should be understood that this may be any of the

naturally occurring bases, *e.g.* adenine, guanine, cytosine, thymine, and uracil, as well as any modified variants thereof or any possible unnatural bases.

When considering the definitions and the known nucleosides (naturally occurring and non-naturally occurring) and nucleoside analogues (including known bi- and tricyclic analogues), it is clear that an oligomer may comprise one or more LNA(s) (which may be identical or different both with respect to the selection of substituent and with respect to selection of biradical) and one or more nucleosides and/or nucleoside analogues. In the present context "oligonucleotide" means a successive chain of nucleosides connected via internucleoside linkages, however, it should be understood that a nucleobase in one or more nucleotide units (monomers) in an oligomer (oligonucleotide) may have been modified with a substituent B as defined above.

The oligomers may be linear, branched or cyclic. In the case of a branched oligomer, the branching points may be located in a nucleoside, in an internucleoside linkage or, in an intriguing embodiment, in an LNA. It is believed that in the latter case, the substituents $R^2$, $R^{2*}$, $R^3$, and $R^{3*}$ may designate two groups P* each designating an internucleoside linkage to a preceding monomer, in particular, one of $R^2$ and $R^{2*}$ designate $P^*$ and one or $R^3$ and $R^{3*}$ designate a further $P^*$.

As mentioned above, the LNA(s) of an oligomer are connected with other monomers via an internucleoside linkage. In the present context, the term "internucleoside linkage" means a linkage consisting of 2 to 4, preferably 3, groups/atoms selected from $-CH_2-$, $-O-$, $-S-$, $-NR^H-$, $>C=O$, $>C=NR^H$, $>C=S$, $-Si(R'')_2-$, $-SO-$, $-S(O)_2-$, $-P(O)_2-$, $-PO(BH_3)-$, $-P(O,S)-$, $-P(S)_2-$, $-PO(R'')-$, $-PO(OCH_3)-$, and $-PO(NHR^H)-$, where $R^H$ is selected form hydrogen and $C_{1-4}$-alkyl, and R'' is selected from $C_{1-6}$-alkyl and phenyl. Illustrative examples of such internucleoside linkages are $-CH_2-CH_2-CH_2-$, $-CH_2-CO-CH_2-$, $-CH_2-CHOH-CH_2-$, $-O-CH_2-O-$, $-O-CH_2-CH_2-$, $-O-CH_2-CH=$ (including $R^5$ when used as a linkage to a succeeding monomer), $-CH_2-CH_2-O-$, $-NR^H-CH_2-CH_2-$, $-CH_2-CH_2-NR^H-$, $-CH_2-NR^H-CH_2-$, $-O-CH_2-CH_2-NR^H-$, $-NR^H-CO-O-$, $-NR^H-CO-NR^H-$, $-NR^H-CS-NR^H-$, $-NR^H-C(=NR^H)-NR^H-$, $-NR^H-CO-CH_2-NR^H-$, $-O-CO-O-$, $-O-CO-CH_2-O-$, $-O-CH_2-CO-O-$, $-CH_2-CO-NR^H-$, $-O-CO-NR^H-$, $-NR^H-CO-CH_2-$, $-O-CH_2-CO-NR^H-$, $-O-CH_2-CH_2-NR^H-$, $-CH=N-O-$, $-CH_2-NR^H-O-$, $-CH_2-O-N=$ (including $R^5$ when used as a linkage to a succeeding monomer), $-CH_2-O-NR^H-$, $-CO-NR^H-CH_2-$, $-CH_2-NR^H-O-$, $-CH_2-NR^H-CO-$, $-O-NR^H-CH_2-$,

-O-$NR^H$-, -O-$CH_2$-S-, -S-$CH_2$-O-, -$CH_2$-$CH_2$-S-, -O-$CH_2$-$CH_2$-S-, -S-$CH_2$-CH= (including $R^5$ when used as a linkage to a succeeding monomer), -S-$CH_2$-$CH_2$-, -S-$CH_2$-$CH_2$-O-, -S-$CH_2$-$CH_2$-S-, -$CH_2$-S-$CH_2$-, -$CH_2$-SO-$CH_2$-, -$CH_2$-$SO_2$-$CH_2$-, -O-SO-O-, -O-$S(O)_2$-O-, -O-$S(O)_2$-$CH_2$-, -O-$S(O)_2$-$NR^H$-, -$NR^H$-$S(O)_2$-$CH_2$-, -O-$S(O)_2$-$CH_2$-, -O-$P(O)_2$-O-, -O-P(O,S)-O-, -O-$P(S)_2$-O-, -S-$P(O)_2$-O-, -S-P(O,S)-O-, -S-$P(S)_2$-O-, -O-$P(O)_2$-S-, -O-P(O,S)-S-, -O-$P(S)_2$-S-, -S-$P(O)_2$-S-, -S-P(O,S)-S-, -S-$P(S)_2$-S-, -O-PO(R'')-O-, -O-$PO(OCH_3)$-O-, -O-$PO(OCH_2CH_3)$-O-, -O-$PO(OCH_2CH_2S$-R)-O-, -O-$PO(BH_3)$-O-, -O-$PO(NHR^N)$-O-, -O-$P(O)_2$-$NR^H$-, -$NR^H$-$P(O)_2$-O-, -O-$P(O,NR^H)$-O-, -$CH_2$-$P(O)_2$-O-, -O-$P(O)_2$-$CH_2$-, and -O-$Si(R'')_2$-O-; among which -$CH_2$-CO-$NR^H$-, -$CH_2$-$NR^H$-O-, -S-$CH_2$-O-, -O-$P(O)_2$-O-, -O-P(O,S)-O-, -O-$P(S)_2$-O-, -$NR^H$-$P(O)_2$-O-, -O-$P(O,NR^H)$-O-, -O-PO(R'')-O-, -O-$PO(CH_3)$-O-, and -O-$PO(NHR^N)$-O-, where $R^H$ is selected form hydrogen and $C_{1-4}$-alkyl, and R'' is selected from $C_{1-6}$-alkyl and phenyl, are especially preferred. Further illustrative examples are given in Mesmaeker et. al., Current Opinion in Structural Biology 1995, 5, 343-355. The left-hand side of the internucleoside linkage is bound to the 5- or 6-membered ring as substituent P', whereas the right-hand side is bound to the 5'-position of a preceding monomer.

It is also clear from the above that the group P may also designate a 5'-terminal group in the case where the LNA in question is the 5'-terminal monomer. Examples of such 5'-terminal groups are hydrogen, hydroxy, optionally substituted $C_{1-6}$-alkyl, optionally substituted $C_{1-6}$-alkoxy, optionally substituted $C_{1-6}$-alkylcarbonyloxy, optionally substituted aryloxy, monophosphate, diphosphate, triphosphate, and -W-A', wherein W is selected from -O-, -S-, and -$N(R^H)$- where $R^H$ is selected from hydrogen and $C_{1-6}$-alkyl, and where A' is selected from DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands (where the latter groups may include a spacer as defined for the substituent B).

In the present description and claims, the terms "monophosphate", "diphosphate", and "triphosphate" mean groups of the formula: -O-$P(O)_2$-$O^-$, -O-$P(O)_2$-O-$P(O)_2$-$O^-$, and -O-$P(O)_2$-O-$P(O)_2$-O-$P(O)_2$-$O^-$, respectively.

In a particularly interesting embodiment, the group P designates a 5'-terminal groups selected from monophosphate, diphosphate and triphosphate. Especially the triphosphate variant is interesting as a substrate

Analogously, the group P* may designate a 3'-terminal group in the case where the LNA in question is the 3'-terminal monomer. Examples of such 3'-terminal groups are hydrogen, hydroxy, optionally substituted $C_{1-6}$-alkoxy, optionally substituted $C_{1-6}$-alkylcarbonyloxy, optionally substituted aryloxy, and -W-A', wherein W is selected from -O-, -S-, and -N($R^H$)- where $R^H$ is selected from hydrogen and $C_{1-6}$-alkyl, and where A' is selected from DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands (where the latter groups may include a spacer as defined for the substituent B).

In a preferred embodiment of the present invention, the oligomer has the following formula V:

$$G\text{-}[Nu\text{-}L]_{n(0)}\text{-}\{[LNA\text{-}L]_{m(q)}\text{-}[Nu\text{-}L]_{n(q)}\}_q\text{-}G^* \quad V$$

wherein

q is 1-50;

each of n(0), .., n(q) is independently 0-10000;

each of m(1), .., m(q) is independently 1-10000;

with the proviso that the sum of n(0), .., n(q) and m(1), .., m(q) is 2-15000;

G designates a 5'-terminal group;

each Nu independently designates a nucleoside selected from naturally occurring nucleosides and nucleoside analogues;

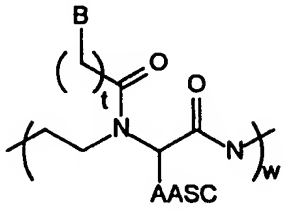
each LNA independently designates a nucleoside analogue;

each L independently designates an internucleoside linkage between two groups selected from Nu and LNA, or L together with G* designates a 3'-terminal group; and

each LNA-L independently designates a nucleoside analogue of the general formula I as defined above, or preferably of the general formula Ia as defined above.

Within this embodiment, as well as generally, the present invention provides the intriguing possibility of including LNAs with different nucleobases, in particular both nucleobases selected from thymine, cytosine and urasil and nucleobases selected from adenine and guanine.

In another embodiment of the present invention, the oligomer further comprises a PNA mono- or oligomer segment of the formula




wherein B is a defined above for the formula I, AASC designates hydrogen or an amino acid side chain, t is 1-5, and w is 1-50.

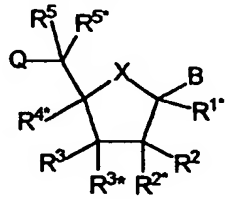
In the present context, the term "amino acid side chain" means a group bound to the α–atom of an α–amino acids, *i.e.* corresponding to the α–amino acid in question without the glycine moiety, preferably an either naturally occurring or a readily available α–amino acid. Illustrative examples are hydrogen (glycine itself), deuterium (deuterated glycine), methyl (alanine), cyanomethyl (β–cyano-alanine), ethyl, 1-propyl (norvaline), 2-propyl (valine), 2-methyl-1-propyl (leucine), 2-hydroxy-2-methyl-1-propyl (β–hydroxy-leucine), 1-butyl (norleucine), 2-butyl (isoleucine), methylthioethyl (methionine), benzyl (phenylalanine), p-amino-benzyl (p-amino-phenylalanine), p-iodo-benzyl (p-iodo-phenylalanine), p-fluoro-benzyl (p-fluoro-phenylalanine), p-bromo-benzyl (p-bromo-phenylalanine), p-chloro-benzyl (p-chloro-phenylalanine), p-nitro-benzyl (p-nitro-phenylalanine), 3-pyridylmethyl (β–(3-pyridyl)-alanine), 3,5-diiodo-4-hydroxy-benzyl (3,5-diiodo-tyrosine), 3,5-dibromo-4-hydroxy-benzyl (3,5-dibromo-tyrosine), 3,5-dichloro-4-hydroxy-benzyl (3,5-dichloro-tyrosine), 3,5-difluoro-4-hydroxy-benzyl (3,5-difluoro-tyrosine), 4-methoxy-benzyl (O-methyl-tyrosine), 2-naphtylmethyl (β–(2-naphtyl)-alanine), 1-naphtylmethyl (β–(1-naphtyl)-alanine), 3-indolylmethyl (tryptophan), hydroxymethyl (serine), 1-hydroxyethyl (threonine), mercaptomethyl (cysteine), 2-mercapto-2-propyl (penicillamine), 4-hydroxybenzyl (tyrosine), amino-carbonylmethyl (asparagine), 2-aminocarbonylethyl (glutamine), carboxymethyl (aspartic acid), 2-carboxyethyl (glutamic acid), aminomethyl (α,β–diaminopropionic acid), 2-aminoethyl (α,γ-diaminobutyric acid), 3-amino-propyl (ornithine), 4-amino-1-butyl (lysine), 3-guanidino-1-propyl (arginine), and 4-imidazolylmethyl (histidine).

PNA mono- or oligomer segment may be incorporated in a oligomer as described in EP 0672677 A2.

The oligomers of the present invention are also intended to cover chimeric oligomers. "Chimeric oligomers" means two or more oligomers with monomers of different origin joined either directly or via a spacer. Illustrative examples of such oligomers which can be combined are peptides, PNA-oligomers, oligomers containing LNA's, and oligonucleotide oligomers.

Apart from the oligomers defined above, the present invention also provides monomeric LNAs useful, *e.g.*, in the preparation of oligomers, as substrates for, *e.g.*, nucleic acid polymerases, polynucleotide kinases, terminal transferases, and as therapeutical agents, see further below. The monomeric LNAs correspond in the overall structure (especially with respect to the possible biradicals) to the LNAs defined as constituents in oligomers, however with respect to the groups P and $P^*$, the monomeric LNAs differ slightly as will be explained below. Furthermore, the monomeric LNAs may comprise functional group protecting groups, especially in the cases where the monomeric LNAs are to be incorporated into oligomers by chemical synthesis.

An interesting subgroup of the possible monomeric LNAs comprises bicyclic nucleoside analogues (LNAs) of the general formula II




II

wherein the substituent B is selected from nucleobases, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands; X is selected from -O-, -S-, $-N(R^{N*})-$, and $-C(R^6R^{6*})-$, preferably from -O-, -S-, and $-N(R^{N*})-$; one of the substituents $R^2$, $R^{2*}$, $R^3$, and $R^{3*}$ is a group Q*;

each of Q and $Q^*$ is independently selected from hydrogen, azido, halogen, cyano, nitro, hydroxy, Prot-O-, Act-O-, mercapto, Prot-S-, Act-S-, $C_{1-6}$-alkylthio, amino, Prot-$N(R^H)$-, Act-$N(R^H)$-, mono- or di($C_{1-6}$-alkyl)amino, optionally substituted $C_{1-6}$-alkoxy,

optionally substituted $C_{1-6}$-alkyl, optionally substituted $C_{2-6}$-alkenyl, optionally substituted $C_{2-6}$-alkenyloxy, optionally substituted $C_{2-6}$-alkynyl, optionally substituted $C_{2-6}$-alkynyloxy, monophosphate, diphosphate, triphosphate, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, ligands, carboxy, sulphono, hydroxymethyl, Prot-O-$CH_2$-, Act-O-$CH_2$-, aminomethyl, Prot-N($R^H$)-$CH_2$-, Act-N($R^H$)-$CH_2$-, carboxymethyl, sulphonomethyl, where Prot is a protection group for -OH, -SH, and -NH($R^H$), respectively, Act is an activation group for -OH, -SH, and -NH($R^H$), respectively, and $R^H$ is selected from hydrogen and $C_{1-6}$-alkyl;

$R^{2*}$ and $R^{4*}$ together designate a biradical selected from -O-, -S-, -N($R^*$)-, -($CR^*R^*$)$_{r+s+1}$-, -($CR^*R^*$)$_r$-O-($CR^*R^*$)$_s$-, -($CR^*R^*$)$_r$-S-($CR^*R^*$)$_s$-, -($CR^*R^*$)$_r$-N($R^*$)-($CR^*R^*$)$_s$-, -O-($CR^*R^*$)$_{r+s}$-O-, -S-($CR^*R^*$)$_{r+s}$-O-, -O-($CR^*R^*$)$_{r+s}$-S-, -N($R^*$)-($CR^*R^*$)$_{r+s}$-O-, -O-($CR^*R^*$)$_{r+s}$-N($R^*$)-, -S-($CR^*R^*$)$_{r+s}$-S-, -N($R^*$)-($CR^*R^*$)$_{r+s}$-N($R^*$)-, -N($R^*$)-($CR^*R^*$)$_{r+s}$-S-, and -S-($CR^*R^*$)$_{r+s}$-N($R^*$)-; $R^2$ and $R^3$ together designate a biradical selected from -O-, -($CR^*R^*$)$_{r+s}$-, -($CR^*R^*$)$_r$-O-($CR^*R^*$)$_s$-, -($CR^*R^*$)$_r$-S-($CR^*R^*$)$_s$-, and -($CR^*R^*$)$_r$-N($R^*$)-($CR^*R^*$)$_s$-; $R^{2*}$ and $R^3$ together designate a biradical selected from -O-, -($CR^*R^*$)$_{r+s}$-, -($CR^*R^*$)$_r$-O-($CR^*R^*$)$_s$-, -($CR^*R^*$)$_r$-S-($CR^*R^*$)$_s$-, and -($CR^*R^*$)$_r$-N($R^*$)-($CR^*R^*$)$_s$-; $R^3$ and $R^{4*}$ together designate a biradical selected from -($CR^*R^*$)$_r$-O-($CR^*R^*$)$_s$-, -($CR^*R^*$)$_r$-S-($CR^*R^*$)$_s$-, and -($CR^*R^*$)$_r$-N($R^*$)-($CR^*R^*$)$_s$-; $R^3$ and $R^5$ together designate a biradical selected from -($CR^*R^*$)$_r$-O-($CR^*R^*$)$_s$-, -($CR^*R^*$)$_r$-S-($CR^*R^*$)$_s$-, and -($CR^*R^*$)$_r$-N($R^*$)-($CR^*R^*$)$_s$-; $R^{1*}$ and $R^{4*}$ together designate a biradical selected from -($CR^*R^*$)$_r$-O-($CR^*R^*$)$_s$-, -($CR^*R^*$)$_r$-S-($CR^*R^*$)$_s$-, and -($CR^*R^*$)$_r$-N($R^*$)-($CR^*R^*$)$_s$-; or $R^{1*}$ and $R^{2*}$ together designate a biradical selected from -($CR^*R^*$)$_r$-O-($CR^*R^*$)$_s$-, -($CR^*R^*$)$_r$-S-($CR^*R^*$)$_s$-, and -($CR^*R^*$)$_r$-N($R^*$)-($CR^*R^*$)$_s$-; wherein $R^*$ is as defined above for the oligomers; and each of the substituents $R^{1*}$, $R^2$, $R^{2*}$, $R^3$, $R^{4*}$, $R^5$, and $R^{5*}$, which are not involved in Q, Q* or the biradical, are as defined above for the oligomers.

It should furthermore be understood, with due consideration of the known bicyclic nucleoside analogues, that $R^2$ and $R^3$ do not together designate a biradical selected from -O-$CH_2$-$CH_2$- and -O-$CH_2$-$CH_2$-$CH_2$-; and $R^3$ and $R^5$ do not together designate a biradical selected from -$CH_2$-$CH_2$-, -O-$CH_2$-, and -O-Si($^i$Pr)$_2$-O-Si($^i$Pr)$_2$-O-.

The monomeric LNAs also comprise basic salts and acid addition salts thereof. Furthermore, it should be understood that any chemical group (including any

nucleobase), which is reactive under the conditions prevailing in chemical oligonucleotide synthesis, is optionally functional group protected as known in the art. This means that groups such as hydroxy, amino, carboxy, sulphono, and mercapto groups, as well as nucleobases, of a monomeric LNA are optionally functional group protected. Protection (and deprotection) is performed by methods known to the person skilled in the art (see, *e.g.*, Greene, T. W. and Wuts, P. G. M., "Protective Groups in Organic Synthesis", 2nd ed., John Wiley, N.Y. (1991), and M.J. Gait, Oligonucleotide Synthesis, IRL Press, 1984).

Illustrative examples of hydroxy protection groups are optionally substituted trityl, such as 4,4'-dimethoxytrityl (DMT), 4-monomethoxytrityl (MMT), and trityl, optionally substituted 9-(9-phenyl)xanthenyl (pixyl), optionally substituted ethoxycarbonyloxy, *p*-phenylazophenyloxycarbonyloxy, tetraahydropyranyl (thp), 9-fluorenylmethoxycarbonyl (Fmoc), methoxytetrahydropyranyl (mthp), silyloxy such as trimethylsilyl (TMS), triisopropylsilyl (TIPS), *tert*-butyldimethylsilyl (TBDMS), triethylsilyl, and phenyldimethylsilyl, benzyloxycarbonyl or substituted benzyloxycarbonyl ethers such as 2-bromo benzyloxycarbonyl, *tert*-butylethers, alkyl ethers such as methyl ether, acetals (including two hydroxy groups), acyloxy such as acetyl or halogen substituted acetyls, *e.g.* chloroacetyl or fluoroacetyl, isobutyryl, pivaloyl, benzoyl and substituted benzoyls, methoxymethyl (MOM), benzyl ethers or substituted benzyl ethers such as 2,6-dichlorobenzyl (2,6-$Cl_2$Bzl). Alternatively, the hydroxy group may be protected by attachment to a solid support optionally through a linker.

Illustrative examples of amino protection groups are Fmoc (fluorenylmethoxycarbonyl), BOC (*tert*-butyloxycarbonyl), trifluoroacetyl, allyloxycarbonyl (alloc, AOC), benzyloxycarbonyl (Z, Cbz), substituted benzyloxycarbonyls such as 2-chloro benzyloxycarbonyl ((2-ClZ), monomethoxytrityl (MMT), dimethoxytrityl (DMT), phthaloyl, and 9-(9-phenyl)xanthenyl (pixyl).

Illustrative examples of carboxy protection groups are allyl esters, methyl esters, ethyl esters, 2-cyanoethylesters, trimethylsilylethylesters, benzyl esters (Obzl), 2-adamantyl esters (O-2-Ada), cyclohexyl esters (OcHex), 1,3-oxazolines, oxazoler, 1,3-oxazolidines, amides or hydrazides.

Illustrative examples of mercapto protecting groups are trityl (Trt), acetamidomethyl (acm), trimethylacetamidomethyl (Tacm), 2,4,6-trimethoxybenzyl (Tmob), *tert*-butylsulfenyl (S*t*Bu), 9-fluorenylmethyl (Fm), 3-nitro-2-pyridinesulfenyl (Npys), and 4-methylbenzyl (Meb).

Furthermore, it may be necessary or desirable to protect any nucleobase included in an monomeric LNA, especially when the monomeric LNA is to be incorporated in an oligomer according to the invention. In the present context, the term "protected nucleobases" means that the nucleobase in question is carrying a protection group selected among the groups which are well-known for a man skilled in the art (see *e.g.* Protocols for Oligonucleotides and Analogs, vol 20, (Sudhir Agrawal, ed.), Humana Press, 1993, Totowa, NJ; S. L. Beaucage and R. P. Iyer, *Tetrahedron*, 1993, 49, 6123; S. L. Beaucage and R. P. Iyer, *Tetrahedron*, 1992, 48, 2223; and E. Uhlmann and A. Peyman, *Chem. Rev.*, 90, 543.). Illustrative examples are benzoyl, isobutyryl, tert-butyl, tert-butyloxycarbonyl, 4-chloro-benzyloxycarbonyl, 9-fluorenylmethyl, 9-fluorenylmethyloxycarbonyl, 4-methoxybenzoyl, 4-methoxytriphenylmethyl, optionally substituted triazolo, *p*-toluenesulphonyl, optionally substituted sulphonyl, isopropyl, optionally substituted amidines, optionally substituted trityl, phenoxyacetyl, optionally substituted acyl, pixyl, tetrahydropyranyl, optionally substituted silyl ethers, and 4-methoxybenzyloxycarbonyl. Chapter 1 in "Protocols for oligonucleotide conjugates", Methods in Molecular Biology, vol 26, (Sudhir Agrawal, ed.), Humana Press, 1993, Totowa, NJ. and S. L. Beaucage and R. P. Iyer, *Tetrahedron*, 1992, 48, 2223 disclose further suitable examples.

In a preferred embodiment, the group B in a monomeric LNA is preferably selected from nucleobases and protected nucleobases.

In an embodiment of the monomeric LNAs according to the present invention, one of Q and Q*, preferably Q*, designates a group selected from Act-O-, Act-S-, Act-N($R^H$)-, Act-O-$CH_2$-, Act-S-$CH_2$-, Act-N($R^H$)-$CH_2$-, and the other of Q and Q*, preferably Q, designates a group selected from hydrogen, azido, halogen, cyano, nitro, hydroxy, Prot-O-, mercapto, Prot-S-, $C_{1-6}$-alkylthio, amino, Prot-N($R^H$)-, mono- or di($C_{1-6}$-alkyl)amino, optionally substituted $C_{1-6}$-alkoxy, optionally substituted $C_{1-6}$-alkyl, optionally substituted $C_{2-6}$-alkenyl, optionally substituted $C_{2-6}$-alkenyloxy, optionally

substituted $C_{2-6}$-alkynyl, optionally substituted $C_{2-6}$-alkynyloxy, monophosphate, diphosphate, triphosphate, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, ligands, carboxy, sulphono, hydroxymethyl, Prot-O-$CH_2$-, aminomethyl, Prot-N($R^H$)-$CH_2$-, carboxymethyl, sulphonomethyl, and $R^H$ is selected from hydrogen and $C_{1-6}$-alkyl.

In the case described above, the group Prot designates a protecting group for -OH, -SH, and -NH($R^H$), respectively. Such protection groups are selected from the same as defined above for hydroxy protection groups, mercapto protection group, and amino protection groups, respectively, however taking into consideration the need for a stable and reversible protection group. However, it is preferred that any protection group for -OH is selected from optionally substituted trityl, such as dimethoxytrityl (DMT), monomethoxytrityl (MMT), and trityl, and 9-(9-phenyl)xanthenyl (pixyl), optionally substituted, tetrahydropyranyl (thp) (further suitable hydroxy protection groups for phosphoramidite oligonucleotide synthesis are described in Agrawal, ed. "Protocols for Oligonucleotide Conjugates"; Methods in Molecular Biology, vol. 26, Humana Press, Totowa, NJ (1994) and Protocols for Oligonucleotides and Analogs, vol 20, (Sudhir Agrawal, ed.), Humana Press, 1993, Totowa, NJ), or protected as acetal; that any protection group for -SH is selected from trityl, such as dimethoxytrityl (DMT), monomethoxytrityl (MMT), and trityl, and 9-(9-phenyl)xanthenyl (pixyl), optionally substituted, tetrahydropyranyl (thp) (further suitable mercapto protection groups for phosphoramidite oligonucleotide synthesis are also described in Agrawal (see above); and that any protecting group for -NH($R^H$) is selected from trityl, such as dimethoxytrityl (DMT), monomethoxytrityl (MMT), and trityl, and 9-(9-phenyl)xanthenyl (pixyl), optionally substituted, tetrahydropyranyl (thp) (further suitable amino protection groups for phosphoramidite oligonucleotide synthesis are also described in Agrawal (see above).

In the embodiment above, as well as for any monomeric LNAs defined herein, Act designates an activation group for -OH, -SH, and -NH($R^H$), respectively. Such activation groups are, *e.g.*, selected from optionally substituted O-phosphoramidite, optionally substituted O-phosphortriester, optionally substituted O-phosphordiester, optionally substituted H-phosphonate, and optionally substituted O-phosphonate.

In the present context, the term "phosphoramidite" means a group of the formula $-P(OR^x)-N(R^y)_2$, wherein $R^x$ designates an optionally substituted alkyl group, *e.g.* methyl, 2-cyanoethyl, or benzyl, and each of $R^y$ designate optionally substituted alkyl groups, *e.g.* ethyl or isopropyl, or the group $-N(R^y)_2$ forms a morpholino group ($-N(CH_2CH_2)_2O$). $R^x$ preferably designates 2-cyanoethyl and the two $R^y$ are preferably identical and designate isopropyl. Thus, an especially relevant phosphoramidite is N,N-diisopropyl-*O*-(2-cyanoethyl)phosphoramidite.

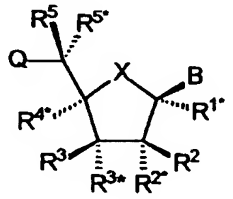
It should be understood that the protecting groups used herein for a single monomeric LNA or several monomeric LNAs may be selected so that when this/these LNA(s) are incorporated in an oligomer according to the invention, it will be possible to perform either a simultaneous deprotection or a sequential deprotection of the functional groups. The latter situation opens for the possibility of regioselectively introducing one or several "active/functional" groups such as DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, where such groups may be attached via a spacer as described above.

In a preferred embodiment, Q is selected from hydrogen, azido, halogen, cyano, nitro, hydroxy, Prot-O-, mercapto, Prot-S-, $C_{1-6}$-alkylthio, amino, Prot-N($R^H$)-, mono- or di($C_{1-6}$-alkyl)amino, optionally substituted $C_{1-6}$-alkoxy, optionally substituted $C_{1-6}$-alkyl, optionally substituted $C_{2-6}$-alkenyl, optionally substituted $C_{2-6}$-alkenyloxy, optionally substituted $C_{2-6}$-alkynyl, optionally substituted $C_{2-6}$-alkynyloxy, monophosphate, diphosphate, triphosphate, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, ligands, carboxy, sulphono, hydroxymethyl, Prot-O-$CH_2$-, aminomethyl, Prot-N($R^H$)-$CH_2$-, carboxymethyl, sulphonomethyl, where Prot is a protection group for -OH, -SH, and -NH($R^H$), respectively, and $R^H$ is selected from hydrogen and $C_{1-6}$-alkyl; and Q* is selected from hydrogen, azido, halogen, cyano, nitro, hydroxy, Act-O-, mercapto, Act-S-, $C_{1-6}$-alkylthio, amino, Act-N($R^H$)-, mono- or di($C_{1-6}$-alkyl)amino, optionally substituted $C_{1-6}$-alkoxy, optionally substituted $C_{1-6}$-alkyl, optionally substituted $C_{2-6}$-alkenyl, optionally substituted $C_{2-6}$-alkenyloxy, optionally substituted $C_{2-6}$-alkynyl, optionally substituted $C_{2-6}$-alkynyloxy, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, ligands, carboxy, sulphono, where

Act is an activation group for -OH, -SH, and -NH($R^H$), respectively, and $R^H$ is selected from hydrogen and $C_{1-6}$-alkyl.

The monomeric LNAs of the general formula II may, as the LNAs incorporated into oligomers, represent various stereoisomers. Thus, the stereochemical variants described above for the LNAs incorporated into oligomers are believed to be equally applicable in the case of monomeric LNAs (however, it should be noted that P should then be replaced with Q).

In a preferred embodiment of the present invention, the monomeric LNA has the general formula IIa




IIa

wherein the substituents are defined as above.

Furthermore, with respect to the definitions of substituents, biradicals, $R^*$, etc. the same preferred embodiments as defined above for the oligomer according to the invention also apply in the case of monomeric LNAs.

In a particularly interesting embodiment of the monomeric LNAs of the present invention, B designates a nucleobase, preferably a nucleobase selected from thymine, cytosine, urasil, adenine and guanine (in particular adenine and guanine), X is -O-, $R^{2*}$ and $R^{4*}$ together designate a biradical selected from -$(CH_2)_{0-1}$-O-$(CH_2)_{1-3}$-, -$(CH_2)_{0-1}$-S-$(CH_2)_{1-3}$-, and -$(CH_2)_{0-1}$-N($R^N$)-$(CH_2)_{1-3}$-, in particular -O-$CH_2$-, -S-$CH_2$- and -$R^N$-$CH_2$-, where $R^N$ is selected from hydrogen and $C_{1-4}$-alkyl, Q designates Prot-O-, $R^{3*}$ is Q* which designates Act-OH, and $R^{1*}$, $R^2$, $R^3$, $R^5$, and $R^{5*}$ each designate hydrogen. In this embodiment, $R^N$ may also be selected from DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups and ligands.

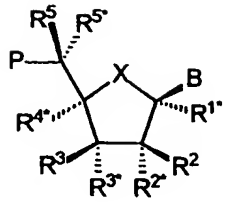
In a further particularly interesting embodiment of the monomeric LNAs of the present invention, B designates a nucleobase, preferably a nucleobase selected from thymine, cytosine, urasil, adenine and guanine (in particular adenine and guanine), X is -O-, $R^{2*}$ and $R^{4*}$ together designate a biradical selected from $-(CH_2)_{0-1}-O-(CH_2)_{1-3}-$, $-(CH_2)_{0-1}-S-(CH_2)_{1-3}-$, and $-(CH_2)_{0-1}-N(R^N)-(CH_2)_{1-3}-$, in particular $-O-CH_2-$, $-S-CH_2-$ and $-R^N-CH_2-$, where $R^N$ is selected from hydrogen and $C_{1-4}$-alkyl, Q is selected from hydroxy, mercapto, $C_{1-6}$-alkylthio, amino, mono- or di($C_{1-6}$-alkyl)amino, optionally substituted $C_{1-6}$-alkoxy, optionally substituted $C_{2-6}$-alkenyloxy, optionally substituted $C_{2-6}$-alkynyloxy, monophosphate, diphosphate, and triphosphate, $R^{3*}$ is Q* which is selected from hydrogen, azido, halogen, cyano, nitro, hydroxy, mercapto, $C_{1-6}$-alkylthio, amino, mono- or di($C_{1-6}$-alkyl)amino, optionally substituted $C_{1-6}$-alkoxy, optionally substituted $C_{1-6}$-alkyl, optionally substituted $C_{2-6}$-alkenyl, optionally substituted $C_{2-6}$-alkenyloxy, optionally substituted $C_{2-6}$-alkynyl, and optionally substituted $C_{2-6}$-alkynyloxy, $R^3$ is selected from hydrogen, optionally substituted $C_{1-6}$-alkyl, optionally substituted $C_{2-6}$-alkenyl, and optionally substituted $C_{2-6}$-alkynyl, and $R^{1*}$, $R^2$, $R^5$, and $R^{5*}$ each designate hydrogen. Also here, $R^N$ may also be selected from DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups and ligands.

In a further particularly interesting embodiment of the monomeric LNAs of the present invention, B designates a nucleobase, X is -O-, $R^2$ and $R^3$ together designate a biradical selected from $-(CH_2)_{0-1}-O-CH=CH-$, $-(CH_2)_{0-1}-S-CH=CH-$, and $-(CH_2)_{0-1}-N(R^N)-CH=CH-$ where $R^N$ is selected from hydrogen and $C_{1-4}$-alkyl, Q is selected from hydroxy, mercapto, $C_{1-6}$-alkylthio, amino, mono- or di($C_{1-6}$-alkyl)amino, optionally substituted $C_{1-6}$-alkoxy, optionally substituted $C_{2-6}$-alkenyloxy, optionally substituted $C_{2-6}$-alkynyloxy, monophosphate, diphosphate, and triphosphate, $R^{3*}$ is Q* which is selected from hydrogen, azido, halogen, cyano, nitro, hydroxy, mercapto, $C_{1-6}$-alkylthio, amino, mono- or di($C_{1-6}$-alkyl)amino, optionally substituted $C_{1-6}$-alkoxy, optionally substituted $C_{1-6}$-alkyl, optionally substituted $C_{2-6}$-alkenyl, optionally substituted $C_{2-6}$-alkenyloxy, optionally substituted $C_{2-6}$-alkynyl, and optionally substituted $C_{2-6}$-alkynyloxy, and $R^{1*}$, $R^{2*}$, $R^{4*}$, $R^5$, and $R^{5*}$ each designate hydrogen.

One aspect of the invention is to provide various derivatives of LNAs for solid-phase and/or solution phase incorporation into an oligomer. As an illustrative example,

monomers suitable for incorporation of (*1S,3R,4R,7S*)-7-hydroxy-1-hydroxymethyl-3-(thymin-1-yl)-2,5-dioxabicyclo[2.2.1]heptane, (*1S,3R,4R,7S*)-7-hydroxy-1-hydroxymethyl-3-(cytosin-1-yl)-2,5-dioxabicyclo[2.2.1]heptane, (*1S,3R,4R,7S*)-7-hydroxy-1-hydroxymethyl-3-(urasil-1-yl)-2,5-dioxabicyclo[2.2.1]heptane, (*1S,3R,4R,7S*)-7-hydroxy-1-hydroxymethyl-3-(guanin-1-yl)-2,5-dioxabicyclo-[2.2.1]heptane, and (*1S,3R,4R,7S*)-7-hydroxy-1-hydroxymethyl-3-(adenin-1-yl)-2,5-dioxabicyclo[2.2.1]heptane using the phosphoramidite approach, the phosphortriester approach, and the *H*-phosphonate approach, respectively, are (*1R,3R,4R,7S*)-7-(2-Cyanoethoxy(diisopropylamino)phosphinoxy)-1-(4,4'-dimethoxytrityloxymethyl)-3-(thymin-1-yl)-2,5-dioxabicyclo[2.2.1]heptane, (*1R,3R,4R,7S*)-7-hydroxy-1-(4,4'-dimethoxytrityloxymethyl)-3-(thymin-1-yl)-2,5-dioxabicyclo[2.2.1]heptane-7-*O*-(2-chlorophenylphosphate), and (*1R,3R,4R,7S*)-7-hydroxy-1-(4,4'-dimethoxytrityloxymethyl)-3-(thymin-1-yl)-2,5-dioxabicyclo[2.2.1]heptane-7-*O*-(*H*-phosphonate) and the 3-(cytosin-1-yl), 3-(urasil-1-yl), 3-(adenin-1-yl) and 3-(guanin-1-yl) analogues thereof, respectively. Furthermore, the analogues where the methyleneoxy biradical of the monomers is substituted with a methylenethio, a methyleneamino, or a 1,2-ethylene biradical are also expected to constitute particularly interesting variants within the present invention. The methylenethio and methyleneamino analogues are believed to equally applicable as the methyleneoxy analogue and therefore the specific reagents corresponding to those mentioned for incorporation of (*1S,3R,4R,7S*)-7-hydroxy-1-hydroxymethyl-3-(thymin-1-yl)-2,5-dioxabicyclo[2.2.1]heptane, (*1S,3R,4R,7S*)-7-hydroxy-1-hydroxymethyl-3-(cytosin-1-yl)-2,5-dioxabicyclo[2.2.1]heptane, (*1S,3R,4R,7S*)-7-hydroxy-1-hydroxymethyl-3-(urasil-1-yl)-2,5-dioxabicyclo[2.2.1]heptane, (*1S,3R,4R,7S*)-7-hydroxy-1-hydroxymethyl-3-(guanin-1-yl)-2,5-dioxabicyclo[2.2.1]heptane, and (*1S,3R,4R,7S*)-7-hydroxy-1-hydroxymethyl-3-(adenin-1-yl)-2,5-dioxabicyclo[2.2.1]heptane should also be considered as particularly interesting reactive monomers within the present invention. For the methyleneamine analogue, it should be noted that the secondary amine may carry a substituent selected from optionally substituted $C_{1-6}$-alkyl such as methyl and benzyl, optionally substituted $C_{1-6}$-alkylcarbonyl such as trifluoroacetyl, optionally substituted arylcarbonyl and optionally substituted heteroarylcarbonyl.

In a particularly interesting embodiment, the present invention relates to an oligomer comprising at least one LNA of the general formula Ia

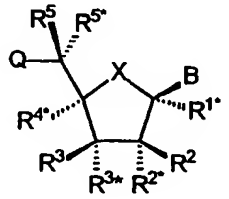


Ia

wherein X is -O-; B is selected from nucleobases, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands; P designates the radical position for an internucleoside linkage to a succeeding monomer, or a 5'-terminal group, such internucleoside linkage or 5'-terminal group optionally including the substituent $R^5$; $R^{3*}$ is a group P* which designates an internucleoside linkage to a preceding monomer, or a 3'-terminal group; $R^{2*}$ and $R^{4*}$ together designate a biradical selected from -O-, -S, -N($R^*$)-, -($CR^*R^*$)$_{r+s+1}$-, -($CR^*R^*$)$_r$-O-($CR^*R^*$)$_s$-, -($CR^*R^*$)$_r$-S-($CR^*R^*$)$_s$-, -($CR^*R^*$)$_r$-N($R^*$)-($CR^*R^*$)$_s$-, -O-($CR^*R^*$)$_{r+s}$-O-, -S-($CR^*R^*$)$_{r+s}$-O-, -O-($CR^*R^*$)$_{r+s}$-S-, -N($R^*$)-($CR^*R^*$)$_{r+s}$-O-, -O-($CR^*R^*$)$_{r+s}$-N($R^*$)-, -S-($CR^*R^*$)$_{r+s}$-S-, -N($R^*$)-($CR^*R^*$)$_{r+s}$-N($R^*$)-, -N($R^*$)-($CR^*R^*$)$_{r+s}$-S-, and -S-($CR^*R^*$)$_{r+s}$-N($R^*$)-; wherein each $R^*$ is independently selected from hydrogen, halogen, azido, cyano, nitro, hydroxy, mercapto, amino, mono- or di($C_{1-6}$-alkyl)amino, optionally substituted $C_{1-6}$-alkoxy, optionally substituted $C_{1-6}$-alkyl, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, and/or two adjacent (non-geminal) $R^*$ may together designate a double bond, and each of r and s is 0-3 with the proviso that the sum r+s is 1-4; each of the substituents $R^{1*}$, $R^2$, $R^3$, $R^5$, and $R^{5*}$ is independently selected from hydrogen, optionally substituted $C_{1-6}$-alkyl, optionally substituted $C_{2-6}$-alkenyl, hydroxy, $C_{1-6}$-alkoxy, $C_{2-6}$-alkenyloxy, carboxy, $C_{1-6}$-alkoxycarbonyl, $C_{1-6}$-alkylcarbonyl, formyl, amino, mono- and di($C_{1-6}$-alkyl)amino, carbamoyl, mono- and di($C_{1-6}$-alkyl)-amino-carbonyl, $C_{1-6}$-alkyl-carbonylamino, carbamido, azido, $C_{1-6}$-alkanoyloxy, sulphono, sulphanyl, $C_{1-6}$-alkylthio, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, and halogen, where two geminal substituents together may designate oxo; and basic salts and acid addition salts thereof. In particular, one $R^*$ is selected from hydrogen, hydroxy, optionally substituted $C_{1-6}$-alkoxy, optionally substituted $C_{1-6}$-alkyl, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, and any remaining substituents $R^*$ are hydrogen.

Especially, the biradical is selected from -O-, $-(CH_2)_{0-1}-O-(CH_2)_{1-3}-$, $-(CH_2)_{0-1}-S-(CH_2)_{1-3}-$, $-(CH_2)_{0-1}-N(R^N)-(CH_2)_{1-3}-$, and $-(CH_2)_{2-4}-$.

In a further particularly interesting embodiment, the present invention relates to an LNA of the general formula IIa



IIa

wherein X is -O-; B is selected from nucleobases, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands; $R^{3*}$ is a group Q*; each of Q and Q* is independently selected from hydrogen, azido, halogen, cyano, nitro, hydroxy, Prot-O-, Act-O-, mercapto, Prot-S-, Act-S-, $C_{1-6}$-alkylthio, amino, Prot-N($R^H$)-, Act-N($R^H$)-, mono- or di($C_{1-6}$-alkyl)amino, optionally substituted $C_{1-6}$-alkoxy, optionally substituted $C_{1-6}$-alkyl, optionally substituted $C_{2-6}$-alkenyl, optionally substituted $C_{2-6}$-alkenyloxy, optionally substituted $C_{2-6}$-alkynyl, optionally substituted $C_{2-6}$-alkynyloxy, monophosphate, diphosphate, triphosphate, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, ligands, carboxy, sulphono, hydroxymethyl, Prot-O-$CH_2$-, Act-O-$CH_2$-, aminomethyl, Prot-N($R^H$)-$CH_2$-, Act-N($R^H$)-$CH_2$-, carboxymethyl, sulphonomethyl, where Prot is a protection group for -OH, -SH, and -NH($R^H$), respectively, Act is an activation group for -OH, -SH, and -NH($R^H$), respectively, and $R^H$ is selected from hydrogen and $C_{1-6}$-alkyl; $R^{2*}$ and $R^{4*}$ together designate a biradical selected from -O-, -S, -N($R^*$)-, $-(CR^*R^*)_{r+s+1}-$, $-(CR^*R^*)_r-O-(CR^*R^*)_s-$, $-(CR^*R^*)_r-S-(CR^*R^*)_s-$, $-(CR^*R^*)_r-N(R^*)-(CR^*R^*)_s-$, $-O-(CR^*R^*)_{r+s}-O-$, $-S-(CR^*R^*)_{r+s}-O-$, $-O-(CR^*R^*)_{r+s}-S-$, $-N(R^*)-(CR^*R^*)_{r+s}-O-$, $-O-(CR^*R^*)_{r+s}-N(R^*)-$, $-S-(CR^*R^*)_{r+s}-S-$, $-N(R^*)-(CR^*R^*)_{r+s}-N(R^*)-$, $-N(R^*)-(CR^*R^*)_{r+s}-S-$, and $-S-(CR^*R^*)_{r+s}-N(R^*)-$; wherein each $R^*$ is independently selected from hydrogen, halogen, azido, cyano, nitro, hydroxy, mercapto, amino, mono- or di($C_{1-6}$-alkyl)amino, optionally substituted $C_{1-6}$-alkoxy, optionally substituted $C_{1-6}$-alkyl, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, and/or two adjacent (non-geminal) $R^*$ may together designate a double bond, and each of r and s is 0-3 with the proviso that the sum r+s is 1-4; each of the substituents $R^{1*}$, $R^2$, $R^3$, $R^5$, and $R^{5*}$ is

independently selected from hydrogen, optionally substituted $C_{1-6}$-alkyl, optionally substituted $C_{2-6}$-alkenyl, hydroxy, $C_{1-6}$-alkoxy, $C_{2-6}$-alkenyloxy, carboxy, $C_{1-6}$-alkoxycarbonyl, $C_{1-6}$-alkylcarbonyl, formyl, amino, mono- and di($C_{1-6}$-alkyl)amino, carbamoyl, mono- and di($C_{1-6}$-alkyl)-amino-carbonyl, $C_{1-6}$-alkyl-carbonylamino, carbamido, azido, $C_{1-6}$-alkanoyloxy, sulphono, sulphanyl, $C_{1-6}$-alkylthio, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, and halogen, where two geminal substituents together may designate oxo; and basic salts and acid addition salts thereof; and with the proviso that any chemical group (including any nucleobase), which is reactive under the conditions prevailing in oligonucleotide synthesis, is optionally functional group protected. Preferably, one $R^*$ is selected from hydrogen, hydroxy, optionally substituted $C_{1-6}$-alkoxy, optionally substituted $C_{1-6}$-alkyl, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, and any remaining substituents $R^*$ are hydrogen. Especially, the biradical is selected from -O-, $-(CH_2)_{0-1}-O-(CH_2)_{1-3}-$, $-(CH_2)_{0-1}-S-(CH_2)_{1-3}-$, $-(CH_2)_{0-1}-N(R^N)-(CH_2)_{1-3}-$, and $-(CH_2)_{2-4}-$.

Generally, the present invention provides oligomers having surprisingly good hybridisation properties with respect to affinity and specificity. Thus, the present invention provides an oligomer comprising at least one nucleoside analogue which imparts to the oligomer a $T_m$ with a complementary DNA oligonucleotide which is at least 2.5 °C higher, preferably at least 3.5 °C higher, in particular at least 4.0 °C higher, especially at least 5.0 °C higher, than that of the corresponding unmodified reference oligonucleotide which does not comprise any nucleoside analogue. In particular, the $T_m$ of the oligomer is at least 2.5 x N °C higher, preferably at least 3.5 x N °C higher, in particular at least 4.0 x N °C higher, especially at least 5.0 x N°C higher, where N is the number of nucleoside analogues.

In the case of hybridisation with a complementary RNA oligonucleotide, the at least one nucleoside analogue imparts to the oligomer a $T_m$ with the complementary DNA oligonucleotide which is at least 4.0 °C higher, preferably at least 5.0 °C higher, in particular at least 6.0 °C higher, especially at least 7.0 °C higher, than that of the corresponding unmodified reference oligonucleotide which does not comprise any nucleoside analogue. In particular, the $T_m$ of the oligomer is at least 4.0 x N °C higher,

preferably at least 5.0 x N °C higher, in particular at least 6.0 x N °C higher, especially at least 7.0 x N°C higher, where N is the number of nucleoside analogues.

The term "corresponding unmodified reference oligonucleotide" is intended to mean an oligonucleotide solely consisting of naturally occurring nucleotides which represents the same nucleobases in the same absolute order (and the same orientation).

The $T_m$ is measured under one of the following conditions (i.e. essentially as illustrated in Example 129):

a) 10mM $Na_2HPO_4$, pH 7.0, 100mM NaCl, 0.1mM EDTA;
b) 10mM $Na_2HPO_4$ pH 7.0, 0.1mM EDTA; or
c) 3M tetrametylammoniumchlorid (TMAC), 10mM $Na_2HPO_4$, pH 7.0, 0.1mM EDTA;

preferably under conditions a), at equimolar amounts (typically 1.0 µM) of the oligomer and the complementary DNA oligonucleotide.

The oligomer is preferably as defined above, where the at least one nucleoside analogue has the formula I where B is a nucleobase. In particular interesting is the cases where at least one nucleoside analogue includes a nucleobase selected from adenine and guanine.

Furthermore, with respect to specificity and affinity, the oligomer, when hybridised with a partially complementary DNA oligonucleotide, or a partially complementary RNA oligonucleotide, having one or more mismatches with said oligomer, should exhibit a reduction in $T_m$, as a result of said mismatches, which is equal to or greater than the reduction which would be observed with the corresponding unmodified reference oligonucleotide which does not comprise any nucleoside analogues. Also, the oligomer should have substantially the same sensitivity of $T_m$ to the ionic strength of the hybridisation buffer as that of the corresponding unmodified reference oligonucleotide.

Oligomers defined herein are typically at least 30% modified, preferably at least 50% modified, in particular 70% modified, and in some interesting applications 100% modified.

The oligomers of the invention has substantially higher 3'-exonucleolytic stability than the corresponding unmodified reference oligonucleotide. This important property can be examined as described in Example 136.

**Definitions**

In the present context, the term "$C_{1-12}$-alkyl" means a linear, cyclic or branched hydrocarbon group having 1 to 12 carbon atoms, such as methyl, ethyl, propyl, *iso*-propyl, cyclopropyl, butyl, *tert*-butyl, *iso*-butyl, cyclobutyl, pentyl, cyclopentyl, hexyl, cyclohexyl, and dodecyl. Analogously, the term "$C_{1-6}$-alkyl" means a linear, cyclic or branched hydrocarbon group having 1 to 6 carbon atoms, such as methyl, ethyl, propyl, *iso*-propyl, pentyl, cyclopentyl, hexyl, cyclohexyl, and the term "$C_{1-4}$-alkyl" is intended to cover linear, cyclic or branched hydrocarbon groups having 1 to 4 carbon atoms, *e.g.* methyl, ethyl, propyl, *iso*-propyl, cyclopropyl, butyl, *iso*-butyl, *tert*-butyl, cyclobutyl.

Preferred examples of "$C_{1-6}$-alkyl" are methyl, ethyl, propyl, *iso*-propyl, butyl, *tert*-butyl, *iso*-butyl, pentyl, cyclopentyl, hexyl, cyclohexyl, in particular methyl, ethyl, propyl, *iso*-propyl, *tert*-butyl, *iso*-butyl and cyclohexyl. Preferred examples of "$C_{1-4}$-alkyl" are methyl, ethyl, propyl, *iso*-propyl, butyl, *tert*-butyl, and *iso*-butyl.

Similarly, the term "$C_{2-12}$-alkenyl" covers linear, cyclic or branched hydrocarbon groups having 2 to 12 carbon atoms and comprising one unsaturated bond. Examples of alkenyl groups are vinyl, allyl, butenyl, pentenyl, hexenyl, heptenyl, octenyl, dodecaenyl. Analogously, the term "$C_{2-6}$-alkenyl" is intended to cover linear, cyclic or branched hydrocarbon groups having 2 to 6 carbon atoms and comprising one unsaturated bond. Preferred examples of alkenyl are vinyl, allyl, butenyl, especially allyl.

Similarly, the term "$C_{2-12}$-alkynyl" means a linear or branched hydrocarbon group having 2 to 12 carbon atoms and comprising a triple bond. Examples hereof are ethynyl, propynyl, butynyl, octynyl, and dodecanyl.

In the present context, *i.e.* in connection with the terms "alkyl", "alkenyl", and "alkynyl", the term "optionally substituted" means that the group in question may be substituted one or several times, preferably 1-3 times, with group(s) selected from hydroxy (which when bound to an unsaturated carbon atom may be present in the tautomeric keto form), $C_{1-6}$-alkoxy (*i.e.* $C_{1-6}$-alkyl-oxy), $C_{2-6}$-alkenyloxy, carboxy, oxo (forming a keto or aldehyde functionality), $C_{1-6}$-alkoxycarbonyl, $C_{1-6}$-alkylcarbonyl, formyl, aryl, aryloxycarbonyl, aryloxy, arylcarbonyl, heteroaryl, heteroaryloxycarbonyl, heteroaryloxy, heteroarylcarbonyl, amino, mono- and di($C_{1-6}$-alkyl)amino; carbamoyl, mono- and di($C_{1-6}$-alkyl)aminocarbonyl, amino-$C_{1-6}$-alkyl-aminocarbonyl, mono- and di($C_{1-6}$-alkyl)amino-$C_{1-6}$-alkyl-aminocarbonyl, $C_{1-6}$-alkylcarbonylamino, cyano, guanidino, carbamido, $C_{1-6}$-alkanoyloxy, sulphono, $C_{1-6}$-alkylsulphonyloxy, nitro, sulphanyl, $C_{1-6}$-alkylthio, halogen, where any aryl and heteroaryl may be substituted as specifically describe below for "optionally substituted aryl and heteroaryl".

Preferably, the substituents are selected from hydroxy, $C_{1-6}$-alkoxy, carboxy, $C_{1-6}$-alkoxycarbonyl, $C_{1-6}$-alkylcarbonyl, formyl, aryl, aryloxycarbonyl, arylcarbonyl, heteroaryl, amino, mono- and di($C_{1-6}$-alkyl)amino, carbamoyl, mono- and di($C_{1-6}$-alkyl)-aminocarbonyl, amino-$C_{1-6}$-alkyl-aminocarbonyl, mono- and di($C_{1-6}$-alkyl)amino-$C_{1-6}$-alkyl-aminocarbonyl, $C_{1-6}$-alkylcarbonylamino, cyano, carbamido, halogen, where aryl and heteroaryl may be substituted 1-5 times, preferably 1-3 times, with $C_{1-4}$-alkyl, $C_{1-4}$-alkoxy, nitro, cyano, amino or halogen. Especially preferred examples are hydroxy, $C_{1-6}$-alkoxy, carboxy, aryl, heteroaryl, amino, mono- and di($C_{1-6}$-alkyl)amino, and halogen, where aryl and heteroaryl may be substituted 1-3 times with $C_{1-4}$-alkyl, $C_{1-4}$-alkoxy, nitro, cyano, amino or halogen.

In the present context the term "aryl" means a fully or partially aromatic carbocyclic ring or ring system, such as phenyl, naphthyl, 1,2,3,4-tetrahydronaphthyl, anthracyl, phenanthracyl, pyrenyl, benzopyrenyl, fluorenyl and xanthenyl, among which phenyl is a preferred example.

The term "heteroaryl" means a fully or partially aromatic carbocyclic ring or ring system where one or more of the carbon atoms have been replaced with heteroatoms, *e.g.* nitrogen (=N- or -NH), sulphur, and/or oxygen atoms. Examples of such heteroaryl groups are oxazolyl, isoxazolyl, thiazolyl, isothiazolyl, pyrrolyl, imidazolyl,

pyrazolyl, pyridinyl, pyrazinyl, pyridazinyl, piperidinyl, coumaryl, furyl, quinolyl, benzothiazolyl, benzotriazolyl, benzodiazolyl, benzooxozolyl, phthalazinyl, phthalanyl, triazolyl, tetrazolyl, isoquinolyl, acridinyl, carbazolyl, dibenzazepinyl, indolyl, benzopyrazolyl, phenoxazonyl.

In the present context, *i.e.* in connection with the terms "aryl" and "heteroaryl", the term "optionally substituted" means that the group in question may be substituted one or several times, preferably 1-5 times, in particular 1-3 times) with group(s) selected from hydroxy (which when present in an enol system may be represented in the tautomeric keto form), $C_{1-6}$-alkyl, $C_{1-6}$-alkoxy, oxo (which may be represented in the tautomeric enol form), carboxy, $C_{1-6}$-alkoxycarbonyl, $C_{1-6}$-alkylcarbonyl, formyl, aryl, aryloxy, aryloxycarbonyl, arylcarbonyl, heteroaryl, amino, mono- and di($C_{1-6}$-alkyl)amino; carbamoyl, mono- and di($C_{1-6}$-alkyl)aminocarbonyl, amino-$C_{1-6}$-alkyl-aminocarbonyl, mono- and di($C_{1-6}$-alkyl)amino-$C_{1-6}$-alkyl-aminocarbonyl, $C_{1-6}$-alkylcarbonylamino, cyano, guanidino, carbamido, $C_{1-6}$-alkanoyloxy, sulphono, $C_{1-6}$-alkylsulphonyloxy, nitro, sulphanyl, dihalogen-$C_{1-4}$-alkyl, trihalogen-$C_{1-4}$-alkyl, halogen, where aryl and heteroaryl representing substituents may be substituted 1-3 times with $C_{1-4}$-alkyl, $C_{1-4}$-alkoxy, nitro, cyano, amino or halogen. Preferred examples are hydroxy, $C_{1-6}$-alkyl, $C_{1-6}$-alkoxy, carboxy, $C_{1-6}$-alkoxycarbonyl, $C_{1-6}$-alkylcarbonyl, aryl, amino, mono- and di($C_{1-6}$-alkyl)amino, and halogen, wherein aryl may be substituted 1-3 times with $C_{1-4}$-alkyl, $C_{1-4}$-alkoxy, nitro, cyano, amino or halogen.

"Halogen" includes fluoro, chloro, bromo, and iodo.

It should be understood that oligomers (wherein LNAs are incorporated) and LNAs as such include possible salts thereof, of which pharmaceutically acceptable salts are especially relevant. Salts include acid addition salts and basic salts. Examples of acid addition salts are hydrochloride salts, sodium salts, calcium salts, potassium salts, etc.. Examples of basic salts are salts where the (remaining) counter ion is selected from alkali metals, such as sodium and potassium, alkaline earth metals, such as calcium, and ammonium ions ($^{+}N(R^g)_3R^h$, where each of $R^g$ and $R^h$ independently designates optionally substituted $C_{1-6}$-alkyl, optionally substituted $C_{2-6}$-alkenyl, optionally substituted aryl, or optionally substituted heteroaryl). Pharmaceutically acceptable salts are, *e.g.*, those described in Remington's Pharmaceutical Sciences,

17. Ed. Alfonso R. Gennaro (Ed.), Mack Publishing Company, Easton, PA, U.S.A., 1985 and more recent editions and in Encyclopedia of Pharmaceutical Technology. Thus, the term "an acid addition salt or a basic salt thereof" used herein is intended to comprise such salts. Furthermore, the oligomers and LNAs as well as any intermediates or starting materials therefor may also be present in hydrate form.

**Preparation of monomers**

In a preferred embodiment, nucleosides containing an additional 2'-*O*,4'-*C*-linked ring were synthesised by the following procedure:

Synthesis of a number of 4'-*C*-hydroxymethyl nucleosides have been reported earlier (R. D. Youssefyeh, J. P. H. Verheyden and J. G. Moffatt, *J. Org. Chem.*, 1979, 44, 1301; G. H. Jones, M. Taniguchi, D. Tegg and J. G. Moffatt, *J. Org. Chem.*, 1979, 44, 1309; C. O-Yang, H. Y. Wu, E. B. Fraser-Smith and K. A. M. Walker, *Tetrahedron Lett.*, 1992, 33, 37; H. Thrane, J. Fensholdt, M. Regner and J. Wengel, *Tetrahedron*, 1995, 51, 10389; K. D. Nielsen, F. Kirpekar, P. Roepstorff and J. Wengel, *Bioorg. Med. Chem.*, 1995, 3, 1493). For exemplification of synthesis of 2'-*O*,4'-*C*-linked bicyclic nucleosides we chose a strategy starting from 4'-*C*-hydroxymethyl furanose derivative **31**. Benzylation, acetylation, and acetolysis followed by another acetylation afforded furanose **33**, a key intermediate for nucleoside coupling. Stereoselective reaction with silylated thymine afforded compound **34** which was deacetylated to give nucleoside diol **35**. Tosylation followed by base-induced ring closure afforded the 2'-*O*,4'-*C*-linked bicyclic nucleoside derivative **36**. Debenzylation yielded the unprotected bicyclic nucleoside analogue **37** which was transformed into the 5'-*O*-4,4'-dimethoxytrityl protected analogue **38** and subsequently into the phosphoramidite derivative **39** for oligonucleotide synthesis. A similar procedure has been used for synthesis of the corresponding uracil, adenine, cytosine and guanine nucleosides as exemplified in the example section. This coupling method is only one of several possible as will be apparent for a person skilled in the art. A strategy starting from a preformed nucleoside is also possible. Thus, for example, conversion of uridine derivative **62** to derivative **44** was successfully accomplished by tosylation, deisopropylidination and base-induced ring-closure. As another example, conversion of nucleoside **67** into nucleoside **61B** has been accomplished as depicted in Figure 34.

Conversion of the bicyclic thymine nucleoside **37** into the corresponding 5-methyl-cytosine nucleoside **65** was accomplished by a known reaction sequence using triazole and $POCl_3$ followed by benzoylation and treatment by ammonia. A similar procedure should be applicable for the synthesis of **57C** from **44**. As another example of possible strategies, coupling of precyclised furanose derivatives already containing an additional ring with nucleobase derivatives is possible. Such a strategy would in addition allow preparation of the corresponding α-nucleoside analogues. When coupling with a protected methyl furanoside of 4-C,2-O-methylene-D-ribofuranose, we obtained mainly a ring-opened product. However, coupling of 1-O-acetyl furanose **207** or thiophenyl furanose **212** yielded successfully LNA nucleosides with the α-anomers as one product. Incorporation of such α-LNA nucleosides will be possible using the standard oligomerisation techniques (as for the LNA oligomers containing Z) yielding α-LNA oligomers. In addition, a synthetic strategy performing nucleoside coupling using a 4'-*C*-hydroxymethyl furanose already activated for ring closure (*e.g.* by containing a mesyl or tosyl group at the 4'-*C*-hydroxymethyl group), is possible as exemplified by conversion of furanose **78** to nucleoside **79** followed by deprotection and ring closure to give **36**. Chemical or enzymatic transglycosylation or anomerisation of appropriate furanose derivatives or nucleosides are yet other possible synthetic strategies. These and other related strategies allow for synthesis of bicyclic nucleosides containing other nucleobases or analogues thereof by either coupling with these nucleobases or analogues, or starting from preformed nucleoside derivatives.

The described examples are meant to be illustrative for the procedures and examples of this invention. The structures of the synthesised compounds were verified using 1D or 2D NMR techniques, *e.g.* NOE experiments.

An additional embodiment of the present invention is to provide bicyclic nucleosides containing additional rings of different sizes and of different chemical structures. From the methods described it is obvious for a person skilled in the art of organic synthesis that cyclisation of other nucleosides is possible using similar procedures, also of nucleosides containing different *C*-branches. The person skilled in the art will be able to find inspiration and guidance for the preparation of substituted nucleoside analogue intermediates in the literature, see *e.g.* WO 96/14329. Regarding rings of different chemical compositions it is clear that using similar procedures or procedures well-

established in the field of organic chemistry, synthesis of for example thio analogues of the exemplified oxo analogues is possible as is the synthesis of the corresponding amino analogues (using for example nucleophilic substitution reactions or reductive alkylations).

In the example section, synthesis of the amino LNA analogues **73-74F** are described. Conversion of **74** and **74D** into standard building blocks for oligomerisation was possible by 5'-*O*-DMT protection and 3'-*O*-phosphitylation following the standard procedures. For the amino LNA analogue, protection of the 2'-amino functionality is needed for controlled linear oligomerisation. Such protection can be accomplished using standard amino group protection techniques like, *e.g.*, Fmoc, trifluoroacetyl or BOC. Alternatively, an N-alkyl group (*e.g.* benzyl, methyl, ethyl, propyl or functionalised alkyl) can be kept on during nucleoside transformations and oligomerisation. In Figures 35 and 36, strategies using N-trifluoroacetyl and N-methyl derivatives are shown. As outlined in Figure 37, conversion of nucleoside **75** into the 2'-thio-LNA nucleoside analogue **76D** has been successfully performed as has the subsequent syntheses of the phosphoramidite derivative **76F**. Compound **76F** has the required structure for automated synthesis of 2'-thio-LNA oligonucleotides. The N-trifluoroacetyl 2'-amino-LNA synthon **74A** has the required structure for automated synthesis of 2'-amino-LNA oligonucleotides.

Synthesis of the corresponding cytosine, guanine, and adenine derivatives of the 2'-thio and 2'-amino LNA nucleosides can be accomplished using strategies analogous to those shown in Figures 35, 36 and 37. Alternative, the stereochemistry around C-2' can be inverted before cyclisations either by using a conveniently configurated, *e.g.* an arabino-configurated, furanose synthon, or by inverting the configuration around the C-2' carbon atom starting from a ribo-configurated nucleoside/furanose. Subsequent activation of the 2'-β-OH, *e.g.* by tosylation, double nucleophilic substitution as in the urasil/thymine example described above, could furnish the desired bicyclic 2'-thio-LNA or 2'-amino-LNA nucleosides. The thus obtained properly protected cytosine, guanine, and adenine analogues can be prepared for oligomerisation using the standard reactions (DMT-protection and phosphitylation) as described above for other examples.

**Preparation of oligomers**

Linear-, branched- (M. Grøtli and B. S. Sproat, *J. Chem. Soc., Chem. Commun.*, 1995, 495; R. H. E. Hudson and M. J. Damha, *J. Am. Chem. Soc.*, 1993, 115, 2119; M. Von Büren, G. V. Petersen, K. Rasmussen, G. Brandenburg, J. Wengel and F. Kirpekar, *Tetrahedron*, 1995, 51, 8491) and circular- (G. Prakash and E. T. Kool, *J. Am. Chem. Soc.*, 1992, 114, 3523) Oligo- and polynucleotides of the invention may be produced using the polymerisation techniques of nucleic acid chemistry well known to a person of ordinary skill in the art of organic chemistry. Phosphoramidite chemistry (S. L. Beaucage and R. P. Iyer, *Tetrahedron*, 1993, 49, 6123; S. L. Beaucage and R. P. Iyer, *Tetrahedron*, 1992, 48, 2223) was used, but *e.g.* H-phosphonate chemistry, phosphortriester chemistry or enzymatic synthesis could also be used. Generally, standard coupling conditions and the phosphoramidite approach was used, but for some monomers of the invention longer coupling time, and/or repeated couplings with fresh reagents, and/or use of more concentrated coupling reagents were used. As another possibility, activators more active than 1H-tetrazole could also be used to increase the rate of the coupling reaction. The phosphoramidietes **39**, **46**, **53**, **57D**, **61D**, and **66** all coupled with satisfactory >95% step-wise coupling yields. An all-phosphorothioate LNA oligomer (Table 7) was synthesised using standard procedures. Thus, by exchanging the normal, *e.g.* iodine/pyridine/$H_2O$, oxidation used for synthesis of phosphordiester oligomers with an oxidation using Beaucage's reagent (commercially available), the phosphorthioate LNA oligomer was efficiently synthesised (stepwise coupling yields > = 98%). The 2'-amino-LNA and 2'methylamino-LNA oligonucleotides (Table 9) were efficiently synthesised (step-wise coupling yields ≥ 98%) using amidites **74A** and **74F**. The 2'-thio-LNA oligonucleotides (Table 8) were efficiently synthesised using amidite **76F** using the standard phosphoramidite procedures as described above for LNA oligonucleotides. After synthesis of the desired sequence, work up was done using standard conditions (cleavage from solid support and removal of protection groups using 30% ammonia for 55 °C for 5 h). Purification of LNA oligonucleotides was done using disposable reversed phase purification cartridges and/or reversed phase HPLC and/or precipitation from ethanol or butanol. Capillary gel electrophoresis, reversed phase HPLC and MALDI-MS was used to verify the purity of the synthesised oligonucleotide analogues,

and to verify that the desired number of bicyclic nucleoside analogues of the invention were incorporated as contemplated.

An additional aspect of the present invention is to furnish procedures for oligonucleotide analogues containing LNA linked by non-natural internucleoside linkages. For example, synthesis of the corresponding phosphorothioate or phosphoramidate analogues is possible using strategies well-established in the field of oligonucleotide chemistry (Protocols for Oligonucleotides and Analogs, vol 20, (Sudhir Agrawal, ed.), Humana Press, 1993, Totowa, NJ; S. L. Beaucage and R. P. Iyer, *Tetrahedron*, 1993, 49, 6123; S. L. Beaucage and R. P. Iyer, *Tetrahedron*, 1992, 48, 2223; E. Uhlmann and A. Peyman, *Chem. Rev.*, 90, 543).

Thus, generally the present invention also provides the use of an LNA as defined herein for the preparation of an LNA modified oligonucleotides. Is should be understood that LNA modified oligonucleotide may comprise normal nucleosides (i.e. naturally occurring nucleosides such as ribonucleosides and/or dioxyribonucleosides), as well as modified nucleosides different from those defined with the general formula II. In a particularly interesting embodiment, incorporation of LNA modulates the ability of the oligonucleotide to act as a substrate for nucleic acid active enzymes.

Furthermore, solid support materials having immobilised thereto an optionally nucleobase protected and optionally 5'-OH protected LNA are especially interesting as material for the synthesis of LNA modified oligonucleotides where an LNA monomer is included in at the 3' end. In this instance, the solid support material is preferable CPG, *e.g.* a readily (commercially) available CPG material onto which a 3'-functionalised, optionally nucleobase protected and optionally 5'-OH protected LNA is linked using the conditions stated by the supplier for that particular material. BioGenex Universial CPG Support (BioGenex, U.S.A.) can e.g. be used. The 5'-OH protecting group may, *e.g.*, be a DMT group. 3'-functional group should be selected with due regard to the conditions applicable for the CPG material in question.

**Applications**

The present invention discloses the surprising finding that various novel derivatives of bicyclic nucleoside monomers (LNAs), when incorporated into oligonucleotides, dramatically increase the affinity of these modified oligonucleotides for both complementary ssDNA and ssRNA compared to the unmodified oligonucleotides. It further discloses the surprising finding that both fully and partly LNA modified oligonucleotides display greatly enhanced hybridisation properties for their complementary nucleic acid sequences. Depending on the application, the use of these LNAs thus offers the intriguing possibility to either greatly increase the affinity of a standard oligonucleotide without compromising specificity (constant size of oligonucleotide) or significantly increase the specificity without compromising affinity (reduction in the size of the oligonucleotide). The present invention also discloses the unexpected finding that LNA modified oligonucleotides, in addition to greatly enhanced hybridisation properties, display many of the useful physicochemical properties of normal DNA and RNA oligonucleotides. Examples given herein include excellent solubility, a response of LNA modified oligonucleotides to salts like sodium chloride and tetramethylammonium chloride which mimic that of the unmodified oligonucleotides, the ability of LNA modified oligonucleotides to act as primers for a variety of polymerases, the ability of LNA modified nucleotides to act as primers in a target amplification reaction using a thermostable DNA polymerase, the ability of LNA modified oligonucleotides to act as a substrate for T4 polynucleotide kinase, the ability of biotinylated LNAs to sequence specifically capture PCR amplicons onto a streptavidine coated solid surface, the ability of immobilised LNA modified oligonucleotides to sequence specifically capture amplicons and very importantly the ability of LNA modified oligonucleotides to sequence specifically target double-stranded DNA by strand invasion. Hence, it is apparent to one of ordinary skills in the art that these novel nucleoside analogues are extremely useful tools to improve the performance in general of oligonucleotide based techniques in therapeutics, diagnostics and molecular biology.

An object of the present invention is to provide monomeric LNAs according to the invention which can be incorporated into oligonucleotides using procedures and equipment well known to one skilled in the art of oligonucleotide synthesis.

Another object of the present invention is to provide fully or partly LNA modified oligonucleotides (oligomers) that are able to hybridise in a sequence specific manner to complementary oligonucleotides forming either duplexes or triplexes of substantially higher affinity than the corresponding complexes formed by unmodified oligonucleotides.

Another object of the present invention is to use LNAs to enhance the specificity of normal oligonucleotides without compromising affinity. This can be achieved by reducing the size (and therefore affinity) of the normal oligonucleotide to an extent that equals the gain in affinity resulting from the incorporation of LNAs.

Another object of the present invention is to provide fully or partly modified oligonucleotides containing both LNAs, normal nucleosides and other nucleoside analogues.

A further object of the present invention is to exploit the high affinity of LNAs to create modified oligonucleotides of extreme affinity that are capable of binding to their target sequences in a dsDNA molecule by way of "strand displacement".

A further object of the invention is to provide different classes of LNAs which, when incorporated into oligonucleotides, differ in their affinity towards their complementary nucleosides. In accordance with the invention this can be achieved by either substituting the normal nucleobases G, A, T, C and U with derivatives having, for example, altered hydrogen bonding possibilities or by using LNAs that differ in their backbone structure. The availability of such different LNAs facilitates exquisite tuning of the affinity of modified oligonucleotides.

Another object of the present invention is to provide LNA modified oligonucleotides which are more resistant to nucleases than their unmodified counterparts.

Another object of the present invention is to provide LNA modified oligonucleotides which can recruit RNAseH.

An additional object of the present invention is to provide LNAs that can act as substrates for DNA and RNA polymerases thereby allowing the analogues to be either incorporated into a growing nucleic acid chain or to act as chain terminators.

A further object of the present invention is to provide LNAs that can act as therapeutic agents. Many examples of therapeutic nucleoside analogues are known and similar derivatives of the nucleoside analogues disclosed herein can be synthesised using the procedures known from the literature (E. De Clercq, *J. Med. Chem.* 1995, **38**, 2491; P. Herdewijn and E. De Clercq: Classical Antiviral Agents and Design og New Antiviral Agents. In: A Textbook of Drug Design and Development; Eds. P. Krogsgaard-Larsen, T. Liljefors and U. Madsen; Harwood Academic Publishers, Amsterdam, 1996, p. 425; I. K. Larsen: Anticancer Agents.In: A Textbook of Drug Design and Development; Eds. P. Krogsgaard-Larsen, T. Liljefors and U. Madsen; Harwood Academic Publishers, Amsterdam, 1996, p. 460).

Double-stranded RNA has been demonstrated to posses anti-viral activity and tumour suppressing activity (Sharp et al., Eur. J. Biochem. 230(1): 97-103, 1995, Lengyel-P. et al., Proc. Natl. Acad. Sci. U.S.A., 90(13): 5893-5, 1993, and Laurent-Crawford et al., AIDS Res. Hum. Retroviruses, 8(2): 285-90, 1992). It is likely that double stranded LNAs may mimic the effect of therapeutically active double stranded RNAs and accordingly such double stranded LNAs has a potential as therapeutic drugs.

When used herein, the term "natural nucleic acid" refers to nucleic acids in the broadest sense, like for instance nucleic acids present in intact cells of any origin or vira or nucleic acids released from such sources by chemical or physical means or nucleic acids derived from such primary sources by way of amplification. The natural nucleic acid may be single, double or partly double stranded, and may be a relatively pure species or a mixture of different nucleic acids. It may also be a component of a crude biological sample containing other nucleic acids and other cellular components. On the other hand, the term "synthetic nucleic acids" refers to any nucleic acid produced by chemical synthesis.

The present invention also provides the use of LNA modified oligonucleotides in nucleic acid based therapeutic, diagnostics and molecular biology. The LNA modified oligonucleotides can be used in the detection, identification, capture, characterisation, quantification and fragmentation of natural or synthetic nucleic acids, and as blocking agents for translation and transcription *in vivo* and *in vitro*. In many cases it will be of interest to attach various molecules to LNA modified oligonucleotides. Such molecules may be attached to either end of the oligonucleotide or they may be attached at one or more internal positions. Alternatively, they may be attached to the oligonucleotide via spacers attached to the 5' or 3' end. Representative groups of such molecules are DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands. Generally all methods for labelling unmodified DNA and RNA oligonucleotides with these molecules can also be used to label LNA modified oligonucleotides. Likewise, all methods used for detecting labelled oligonucleotides generally apply to the corresponding labelled, LNA modified oligonucleotides.

*Therapy*

The term "strand displacement" relates to a process whereby an oligonucleotide binds to its complementary target sequence in a double stranded DNA or RNA so as to displace the other strand from said target strand.

In an aspect of the present invention, LNA modified oligonucleotides capable of performing "strand displacement" are exploited in the development of novel pharmaceutical drugs based on the "antigene" approach. In contrast to oligonucleotides capable of making triple helices, such "strand displacement" oligonucleotides allow any sequence in a dsDNA to be targeted and at physiological ionic strength and pH.

The "strand displacing" oligonucleotides can also be used advantageously in the antisense approach in cases where the RNA target sequence is inaccessible due to intramolecular hydrogen bonds. Such intramolecular structures may occur in mRNAs and can cause significant problems when attempting to "shut down" the translation of the mRNA by the antisense approach.

Other classes of cellular RNAs, like for instance tRNAs, rRNAs snRNAs and scRNAs, contain intramolecular structures that are important for their function. These classes of highly structured RNAs do not encode proteins but rather (in the form of RNA/protein particles) participate in a range of cellular functions such as mRNA splicing, polyadenylation, translation, editing, maintainance of chromosome end integrity, etc.. Due to their high degree of structure, that impairs or even prevent normal oligonucleotides from hybridising efficiently, these classes of RNAs has so far not attracted interest as antisense targets.

The use of high affinity LNA monomers should facilitate the construction of antisense probes of sufficient thermostability to hybridise effectively to such target RNAs. Therefore, in a preferred embodiment, LNA is used to confer sufficient affinity to the oligonucleotide to allow it to hybridise to these RNA classes thereby modulating the qualitative and/or quantitative function of the particles in which the RNAs are found.

In some cases it may be advantageous to down-regulate the expression of a gene whereas in other cases it may be advantageous to activate it. As shown by Møllegaard et al. (Møllegaard, N. E.; Buchardt, O.; Egholm, M.; Nielsen, P. E. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 3892), oligomers capable of "strand displacement" can function as RNA transcriptional activators. In an aspect of the present invention, the LNAs capable of "strand displacement" are used to activate genes of therapeutic interest.

In chemotherapy of numerous viral infections and cancers, nucleosides and nucleoside analogues have proven effective. LNA nucleosides are potentially useful as such nucleoside based drugs.

Various types of double-stranded RNAs inhibit the growth of several types of cancers. Duplexes involving one or more LNA oligonucleotide(s) are potentially useful as such double-stranded drugs.

The invention also concerns a pharmaceutical composition comprising a pharmaceutically active LNA modified oligonucleotide or a pharmaceutically active

LNA monomer as defined above in combination with a pharmaceutically acceptable carrier.

Such compositions may be in a form adapted to oral, parenteral (intravenous, intraperitoneal), intramuscular, rectal, intranasal, dermal, vaginal, buccal, ocularly, or pulmonary administration, preferably in a form adapted to oral administration, and such compositions may be prepared in a manner well-known to the person skilled in the art, e.g. as generally described in "Remington's Pharmaceutical Sciences", 17. Ed. Alfonso R. Gennaro (Ed.), Mark Publishing Company, Easton, PA, U.S.A., 1985 and more recent editions and in the monographs in the "Drugs and the Pharmaceutical Sciences" series, Marcel Dekker.

*Diagnostics*

Several diagnostic and molecular biology procedures have been developed that utilise panels of different oligonucleotides to simultaneously analyse a target nucleic acid for the presence of a plethora of possible mutations. Typically, the oligonucleotide panels are immobilised in a predetermined pattern on a solid support such that the presence of a particular mutation in the target nucleic acid can be revealed by the position on the solid support where it hybridises. One important prerequisite for the successful use of panels of different oligonucleotides in the analysis of nucleic acids is that they are all specific for their particular target sequence under the single applied hybridisation condition. Since the affinity and specificity of standard oligonucleotides for their complementary target sequences depend heavily on their sequence and size this criteria has been difficult to fulfil so far.

In a preferred embodiment, therefore, LNAs are used as a means to increase affinity and/or specificity of the probes and as a means to equalise the affinity of different oligonucleotides for their complementary sequences. As disclosed herein such affinity modulation can be accomplished by, *e.g.*, replacing selected nucleosides in the oligonucleotide with an LNA carrying a similar nucleobase. As further shown herein, different classes of LNAs exhibit different affinities for their complementary nucleosides. For instance, the 2-3 bridged LNA with the T-nucleobase exhibits less affinity for the A-nucleoside than the corresponding 2-4 bridged LNA. Hence, the use

of different classes of LNAs offers an additional level for fine-tuning the affinity of a oligonucleotide.

In another preferred embodiment the high affinity and specificity of LNA modified oligonucleotides is exploited in the sequence specific capture and purification of natural or synthetic nucleic acids. In one aspect, the natural or synthetic nucleic acids are contacted with the LNA modified oligonucleotide immobilised on a solid surface. In this case hybridisation and capture occurs simultaneously. The captured nucleic acids may be, for instance, detected, characterised, quantified or amplified directly on the surface by a variety of methods well known in the art or it may be released from the surface, before such characterisation or amplification occurs, by subjecting the immobilised, modified oligonucleotide and captured nucleic acid to dehybridising conditions, such as for example heat or by using buffers of low ionic strength.

The solid support may be chosen from a wide range of polymer materials such as for instance CPG (controlled pore glass), polypropylene, polystyrene, polycarbonate or polyethylene and it may take a variety of forms such as for instance a tube, a micro-titer plate, a stick, a bead, a filter, etc.. The LNA modified oligonucleotide may be immobilised to the solid support via its 5' or 3' end (or via the terminus of linkers attached to the 5' or 3' end) by a variety of chemical or photochemical methods usually employed in the immobilisation of oligonucleotides or by non-covalent coupling such as for instance via binding of a biotinylated LNA modified oligonucleotide to immobilised streptavidin. One preferred method for immobilising LNA modified oligonucleotides on different solid supports is photochemical using a photochemically active anthraquinone covalently attached to the 5' or 3' end of the modified oligonucleotide (optionally via linkers) as described in (WO 96/31557). Thus, the present invention also provide a surface carrying an LNA modified oligonucleotide.

In another aspect the LNA modified oligonucleotide carries a ligand covalently attached to either the 5' or 3' end. In this case the LNA modified oligonucleotide is contacted with the natural or synthetic nucleic acids in solution whereafter the hybrids formed are captured onto a solid support carrying molecules that can specifically bind the ligand.

In still another aspect, LNA modified oligonucleotides capable of performing "strand displacement" are used in the capture of natural and synthetic nucleic acids without prior denaturation. Such modified oligonucleotides are particularly useful in cases where the target sequence is difficult or impossible to access by normal oligonucleotides due to the rapid formation of stable intramolecular structures. Examples of nucleic acids containing such structures are rRNA, tRNA, snRNA and scRNA.

In another preferred embodiment, LNA modified oligonucleotides designed with the purpose of high specificity are used as primers in the sequencing of nucleic acids and as primers in any of the several well known amplification reactions, such as the PCR reaction. As shown herein, the design of the LNA modified oligonucleotides determines whether it will sustain a exponential or linear target amplification. The products of the amplification reaction can be analysed by a variety of methods applicable to the analysis of amplification products generated with normal DNA primers. In the particular case where the LNA modified oligonucleotide primers are designed to sustain a linear amplification the resulting amplicons will carry single stranded ends that can be targeted by complementary probes without denaturation. Such ends could for instance be used to capture amplicons by other complementary LNA modified oligonucleotides attached to a solid surface.

In another aspect, LNA modified oligos capable of "strand displacement" are used as primers in either linear or exponential amplification reactions. The use of such oligos is expected to enhance overall amplicon yields by effectively competing with amplicon re-hybridisation in the later stages of the amplification reaction. Demers, et al. (Nucl. Acid Res. 1995, Vol 23, 3050-3055) discloses the use of high-affinity, non-extendible oligos as a means of increasing the overall yield of a PCR reaction. It is believed that the oligomers elicit these effect by interfering with amplicon re-hybridisation in the later stages of the PCR reaction. It is expected that LNA modified oligos blocked at their 3' end will provide the same advantage. Blocking of the 3' end can be achieved in numerous ways like for instance by exchanging the 3' hydroxyl group with hydrogen or phosphate. Such 3' blocked LNA modified oligos can also be used to selectively amplify closely related nucleic acid sequences in a way similar to that described by Yu et al. (Biotechniques, 1997, 23, 714-716).

In recent years, novel classes of probes that can be used in for example real-time detection of amplicons generated by target amplification reactions have been invented. One such class of probes have been termed "Molecular Beacons". These probes are synthesised as partly self-complementary oligonucleotides containing a fluorophor at one end and a quencher molecule at the other end. When free in solution the probe folds up into a hairpin structure (guided by the self-complimentary regions) which positions the quencher in sufficient closeness to the fluorophor to quench its fluorescent signal. Upon hybridisation to its target nucleic acid, the hairpin opens thereby separating the fluorophor and quencher and giving off a fluorescent signal.

Another class of probes have been termed "Taqman probes". These probes also contain a fluorophor and a quencher molecule. Contrary to the Molecular Beacons, however, the quenchers ability to quench the fluorescent signal from the fluorophor is maintained after hybridisation of the probe to its target sequence. Instead, the fluorescent signal is generated after hybridisation by physical detachment of either the quencher or fluorophor from the probe by the action of the 5´exonuxlease activity of a polymerase which has initiated synthesis from a primer located 5´ to the binding site of the Taqman probe.

High affinity for the target site is an important feature in both types of probes and consequently such probes tends to be fairly large (typically 30 to 40 mers). As a result, significant problems are encountered in the production of high quality probes. In a preferred embodiment, therefore, LNA is used to improve production and subsequent performance of Taqman probes and Molecular Beacons by reducing their size whilst retaining the required affinity.

In a further aspect, LNAs are used to construct new affinity pairs (either fully or partially modified oligonucleotides). The affinity constants can easily be adjusted over a wide range and a vast number of affinity pairs can be designed and synthesised. One part of the affinity pair can be attached to the molecule of interest (*e.g.* proteins, amplicons, enzymes, polysaccharides, antibodies, haptens, peptides, PNA, etc.) by standard methods, while the other part of the affinity pair can be attached to *e.g.* a solid support such as beads, membranes, micro-titer plates, sticks, tubes, etc. The solid support may be chosen from a wide range of polymer materials such as for

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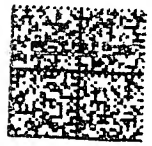

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